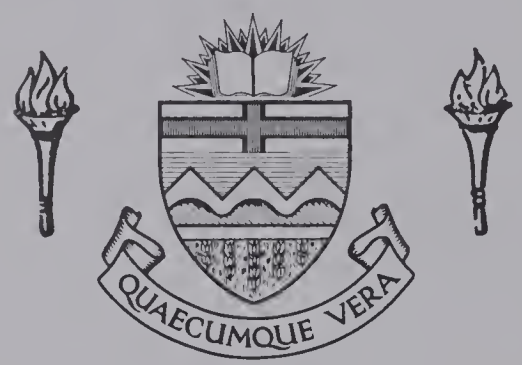

THE UNIVERSITY OF ALBERTA

THE CALCIUM MOVEMENTS DURING EXCITATION CONTRACTION COUPLING IN VASCULAR SMOOTH MUSCLE AND THEIR DETECTION USING LANTHANUM AS A TOOL

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE CALCIUM MOVEMENTS DURING EXCITATION CONTRACTION COUPLING IN VASCULAR SMOOTH MUSCLE AND THEIR DETECTION USING LANTHANUM AS A TOOL submitted by DAVID JOHN FREEMAN in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Studies on the effects of Lanthanum (La) on Ca^{45} movements in rabbit aorta revealed that addition of La (2mM) during the course of Ca^{45} efflux from aortic rings increased the rate of loss of Ca^{45} from the tissue, presumably by promoting the liberation of extracellularly bound Ca^{45}. However, tissue rings washed in La solutions for extended periods of time eventually exhibited higher residual Ca^{45} content than controls washed in the absence of La. This suggests that La inhibits the escape of Ca^{45} from within the cell concurrent with the loss of extracellularly bound Ca^{45}. Tissue rings exposed to La-containing Ca^{45} labelled normal, high potassium (HK) and Noradrenaline (NA) solutions and washed in La solution, exhibited decreased residual Ca^{45} compared with similarly washed controls incubated in the absence of La. This result suggests that La blocks the entrance of Ca^{45} into the cell.

The conclusions reached from the results of this study are in accord with the assumptions made by van Breemen and co-workers (van Breemen 1969, van Breemen and De Weer, 1970) that La displaces extracellular bound Ca while inhibiting the influx and efflux of cellular Ca^{45}. In addition, the data indicate that La (2mM) inhibits the influx of Ca^{45} almost completely, but La at this concentration, only partially blocks the efflux of Ca^{45} from intracellular sites.

The development of tension in rabbit aorta occurs in two stages: an initial rapid or phasic contraction followed by a tonic contraction.

Exposure to La (2mM) prior to stimulation prevented both stages of the contracture to HK. La also relaxed the HK contracture when added during the tonic stage of this response; thereafter a phasic response to NA could still be elicited. La did not prevent the NA contracture fully; an initial phasic response could be achieved, i.e., the tonic stage of the contracture was abolished. La added during the phasic stage of the NA contracture caused relaxation. These actions of La suggest that an influx of Ca from extracellular sources maintains both the phasic and tonic stages of th HK contracture and the tonic stage of the NA response, but that the NA phasic response is sustained by a release of intracellular Ca.

Using La to disclose small movements of intracellular Ca^{45} revealed that HK stimulated an increased uptake of Ca^{45}, but that NA had a much smaller effect. Although these results are consistent with a larger influx of extracellular Ca^{45} during HK than during NA stimulation, there was no simple correlation between tension developed and the uptake of Ca^{45} by these tissues.

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I. INTRODUCTION

In 1883, Ringer observed that calcium (Ca) ions were required to maintain the mechanical activity of the heart. Since that time, Ca has been shown to be necessary for mechanical activity of all types of muscle. The role of Ca in excitation-contraction coupling in skeletal muscle has been extensively studied and a reasonably clear understanding of the processes involved in contraction has emerged (Sandow, 1965; Bianchi, 1968). On the other hand, such studies in smooth muscle are less extensive, and the mode of action of Ca in excitation-contraction coupling is poorly understood (Somlyo and Somlyo, 1968; Bohr, 1964; Daniel, Sehdev and Robinson, 1962). In many properties, smooth muscle contractile machinery and its reaction to divalent cations are similar to those of striated muscle but there are apparent differences in excitation-contraction coupling and associated Ca mobilization in these two types of muscle. These similarities and differences will be mentioned in subsequent sections of this review.

A. Contractile Proteins

Based on histological observations, muscle has been characterized by the adjectives 'striated' and 'smooth'. The former type of muscle or striated muscle has been investigated extensively both by light and electron microscopy. These investigations revealed that the light and dark striations are the result of an orderly arrangement of thin (actin) filaments and thick (myosin) filaments aligned parallel to the long axis of the muscle fibre. Lying in the centre region of each contractile unit or 'sarcomere' is a dark band that has been shown to be a region of overlap and cross-bridging between actin and myosin filaments. Cross section of this region of overlap revealed a hexagonal arrangement of six actin filaments surrounding a central myosin filament. Huxley (1957) has proposed that tension and shortening is developed by a relative movement of these filaments (Sliding Filament Theory) and in general, this theory has been accepted as a mechanism of contraction in striated muscle.

As implied by the adjective 'smooth', smooth muscle contains no observable orderly sequence of light and dark bands as seen in striated muscle. The absence of these bands appears not to be due to lack of actin and myosin filaments but rather to a disorganized spacial arrangement of these filaments. Up until 1968 electron microscopic studies revealed one type of filament in smooth muscle (Hanson and Lowy, 1957; Shoenberg, 1965). This filament was thought to be actin because of its similar dimensions to the actin filament of skeletal muscle. Myosin filaments were not observed in these studies.

In 1968, Nonomura, using the 'block staining' technique was able to distinguish two types of filament in freshly prepared taenia coli, but these were distributed in a disorderly manner and no definite relationship could be observed between the two types of thick and thin filaments. In certain cases, thin filaments were seen lying alongside thick filaments with an apparent bridge between the two. More recent studies of a variety of smooth muscles, including vascular smooth muscle, clearly indicate the presence of thick and thin filaments that were in some cases observed in closely associated hexagonal or circular arrays (Rice, Moses, McManus, Brady and Blasick, 1970; Somlyo, Devine and Somlyo, 1971).

The electron microscopic evidence for actin and myosin filaments mentioned above supports the growing contention that the mechanism by which smooth and striated muscle contract is similar. Furthermore, studies of the mechanical properties of smooth muscle have revealed optical, thermal and length-tension, force-velocity relationships that are very similar to those of striated muscle and consistent with the Sliding Filament hypothesis (Abbott and Lowy, 1955; 1956; Somlyo and Somlyo, 1964; Gordon and Siegman, 1971).

The realization that similarities did exist between striated muscle protein and smooth muscle protein came about through the work of Csapo (1949). He demonstrated that the properties of protein extracted from uterine smooth muscle showed fundamental similarities to skeletal actomyosin as measured by their viscous behavior, sedimentation rate and electrophoretic mobility. An addition of adenosine triphosphate (ATP) to the extract led to dissociation of the original

protein to two constituent proteins which were later shown to have identical properties to skeletal actin and myosin (Cohen, Lowy and Kucera, 1961; Needham and Williams, 1959). Furthermore, uterine myosin was shown to combine with skeletal actin and uterus actin with skeletal myosin (Needham, 1959). Likewise, vascular actin and skeletal myosin combine to form actomyosin (Ruegg, Strassner, Shirmer, 1965).

Unlike striated muscle actomyosin, smooth muscle protein is extracted at low ionic strength and the yield approximately one tenth of that obtained from skeletal muscle (Needham and Williams, 1959). At low ionic strengths smooth muscle myosin does not aggregate into visible filaments, as does skeletal muscle myosin, but may form dimers (Shoenberg, Ruegg, Needham, Schirmer and Gansler, 1966). It has therefore been suggested that the lack of myosin aggregation may account for the difficulty in visualizing myosin filaments electron microscopically (Shoenberg, 1969; Shirmer, 1965). Furthermore, the demonstration that myosin filaments could be obtained in homogenate containing magnesium (Mg) and ATP with trace amounts of Ca (but not in those containing Mg, ATP, Ca or Ca + ATP) has led to the suggestion that myosin only aggregates into a visible filament following excitation and release of Ca at the onset of contraction (Shoenberg, 1969). However, this seems unlikely in light of the recent reports of the presence of thick and thin filaments in both relaxed and contracted smooth muscle (Rice et al., 1970; Somlyo et al., 1971).

Similar to skeletal muscle proteins, smooth muscle myosin ATPase is localized in the heavy meromyosin portion of this molecule and is activated by Ca. Furthermore, activation can be inhibited by

Mg (Gaspar-Godfroid, 1964; Murphy, Bohr and Newman, 1969). In glycerinated smooth muscle in which the contractile proteins are in direct contact with extracellular medium the threshold calcium concentration for contraction has been shown to be approximately 10^{-7}M (Filo, Bohr and Ruegg, 1965) and in vascular smooth muscle extracts, maximum ATPase activity is achieved with 10^{-5}M calcium (Sparrow, Maxwell and Ruegg, 1970).

Tropomyosin, a complex of tropomyosin B and troponin is associated with actin in crude skeletal extracts of actomyosin (Ebashi, 1966). Both troponin and tropomyosin B are necessary for Ca sensitivity of crude proteins,since neither protein alone is capable of conferring Ca sensitivity on purified actomyosin (Wideman, Maruyama and Hagashi, 1970) but troponin has been suggested as the actual receptor protein to which Ca binds to initiate superprecipitation of skeletal actomyosin (Ebashi, Ebashi and Kodama,1967; Fuchs and Briggs, 1968). It appears that tropomyosin also exists in crude extracts of smooth muscle (Ebashi, Iwakura, Nakajima, Nakamura and Choi, 1966; Murphy, Bohr and Newman, 1969). It can be removed as in skeletal extracts, by purification of the crude extract and this consequently leads to loss of Ca-induced superprecipitation (Ebashi et al., 1966; Murphy et al., 1969).

The foregoing discussion has indicated briefly that contractile proteins in smooth and striated muscle are functionally similar and it is becoming increasingly clear that the 'Sliding Filament Theory' of contraction, first applied to striated muscle can equally well be

applied to smooth muscle and that Ca plays an important role in this mechanism in both muscle types.

B. Membrane Electrical Phenomenon in Smooth Muscle and its Role in Excitation contraction coupling

A comparison of the electrical events in skeletal muscle and smooth muscle membranes suggests that electrical depolarization of the membrane is not a necessary prerequisite for tension development induced by the neurotransmitters, noradrenaline (NA) or acetylcholine (Ach). In skeletal muscle, Ach and carbachol contractions were abolished in sodium (Na)-free solutions (Frank, 1963). Furthermore, neither Ach nor carbachol could cause a mechanical response in muscle depolarized by 95mM K_2SO_4 or 123mM KCl. Yet in vascular smooth muscle, it has been reported that NA contractures, although moderately reduced, can still be elicited in Na-free solutions (Briggs, 1962; Hiraoka, Yamagishi and Sano, 1968) and also drug contractures can be obtained in both spontaneously active and quiescent depolarized smooth muscle (Evans, Thesleff and Schild, 1958; Edman and Schild, 1961; Johansson, Jonsson, Axelsson and Wahlstrom, 1967). Attempts to determine the relationship of electrical events to mechanical events during stimulation of vascular smooth muscle have yielded various results.

Bar (1961), using wick electrodes observed no change in membrane electrical activity during NA contractions of the carotid artery. Similar results were reported by Su et al. (1964) using intracellular electrodes. On the other hand, Shibata and Briggs (1966), who also used microelectrodes, reported hyperpolarization to NA. Nevertheless both intracellular electrode studies revealed depolarization during high K^+ contractions. Measurement of electrical changes with the sucrose

gap have detected depolarization accompanied by contraction in both spontaneously active (mesenteric vein) and quiescent (pulmonary artery) vascular smooth muscle (Keating, 1964; Somlyo and Somlyo, 1968). In the latter study contractile activity was generally correlated with the degree of depolarization but even so maximum tension was not accompanied by maximal depolarization and in fact depolarization with 179mM K^+ was greater than that with NA even though the tension with K^+ was smaller than that produced by NA. More recently similar results have been obtained using intracellular electrodes (Somlyo, Vinall, Somlyo, 1969).

The observation that electrical activity, be it the graded depolarization of quiescent smooth muscle (Somlyo and Somlyo, 1968) or the increased spike frequency of spontaneously active smooth muscle (Cuthbert and Sutter, 1965; Johansson et al., 1967) is only "generally" correlated with tension development and the reports that drugs stimulate increased tension development in K^+ depolarized smooth muscle implies that drug action is not exclusively associated with or controlled by a change in transmembrane potential. This apparent non-electrical excitation-contraction-coupling has been termed 'Pharmacomechanical' by Somlyo (1968) as opposed to electromechanical coupling exhibited in skeletal muscle. In skeletal muscle, pharmacomechanical coupling can be induced with caffeine which occurs without membrane depolarization (Axelsson and Thesleff, 1958).

Studies on the ionic basis of the electrical activity in spontaneously active smooth muscle particularly that of the taenia coli, suggest that not only Na but also Ca carries the spike producing current.

The reduction of spiking activity in Na deficient medium can be offset by increasing the external Ca concentration (Bülbring and Kuriyama, 1963; Brading, Bülbring and Tomita, 1969). Furthermore, tetrodotoxin which usually inhibits Na conductance selectively is ineffective in reducing the spike activity (Kuriyama, Osa and Toida, 1966). It has been suggested that Ca may only substitute for Na when the latter is deficient (Bülbring and Kuriyama, 1963); however, since reduction of Na reduces maximal rate of rise of the spike (Holman, 1958) suggesting a decreased inward current, it appears that both Na and Ca may make up the depolarizing current during spike activity. Similarly Ca has been implicated as a current carrier in uterine and vascular smooth muscle. Action potentials can be generated in rat myometrium bathed in Na deficient solutions (Daniel and Singh, 1958) and recent evidence indicates that Ca is more important than Na in determining the maximum rate of rise and amplitude of both spontaneous (Marshall, 1965) and electrically stimulated spikes (Abe, 1968).

In vascular smooth muscle it has been shown that Ca depletion of the mesenteric vein abolishes both spiking and mechanical activity; however, introduction of NA after spiking was abolished induced a return of both spiking and mechanical responses. These responses to NA could be prevented by inclusion of EGTA in the bathing medium prior to NA stimulation (Johansson et al., 1967). The authors suggest that NA releases bound Ca which participates in both spike current and activation of contractile proteins. Calculation of the amount of Ca required to carry the charge associated with spike current across the taenia coli cell membrane have revealed values in keeping with this

suggestion. One spike would contribute per kg of tissue approximately 0.004 mMoles Ca (Casteels, 1970) or 0.005 mMoles Ca (Goodford, 1970) which would raise the intracellular Ca concentration by 7×10^{-6} M on the basis of a cell volume of 6×10^{-7} 1/mg tissue (Casteels, 1970). This rise in intracellular Ca would be adequate to initiate mechanical activity.

C. Calcium Pools in Smooth Muscle

The reported Ca concentrations in smooth muscle vary between 4.2 mM/kg wet weight in the guinea pig ileum (Schatzmann, 1961) to 1.4 mM/kg wet weight in the cat small intestine (Sperelakis, 1962). Tissue Ca content has been shown to vary with the condition of the muscle (Bauer, Goodford and Huter, 1965) with the concentration of the incubation medium (Schatzman, 1961; Bauer et al., 1965; van Breemen, Daniel and van Breemen, 1966) and with cooling of the muscle (Bauer et al., 1965; Potter and Sparrow, 1968). Therefore, the functional state of the muscle is of prime consideration when interpreting Ca content determinations. The effect of stimulant drugs and depolarization solutions is apparently to increase intracellular free Ca to a level which would activate contractile proteins. This movement of Ca is very small, i.e. 10^{-6} M/kg compared to total tissue Ca with the result that difficulty has arisen in interpreting results of experiments specifically designed to measure Ca movements during excitation-contraction-coupling.

van Breemen and Daniel (1966) were unable to show that either high potassium (HK) or Ach produced an increase in Ca^{45} uptake in rat uterus. Similar results were reported by Schatzman (1964) in the taenia coli stimulated with HK, Ach and carbachol, but in contrast Urakawa and Holland (1964) reported increased Ca^{45} uptake as well as a net gain of total tissue Ca in the same tissue. It is questionable that the increase in total Ca was connected with contraction due to the fact that Ca content increased at a time after constant tension

had been reached (Hurwitz, Joiner and Von Hagen, 1967). In vascular smooth muscle increased Ca^{45} uptake during contracture has been reported by Briggs and Melvin (1961) and Briggs (1962). In the former study a 107% increase in uptake of Ca^{45} was observed with NA stimulation while a 225% increase was observed after incubation in low sodium medium. Likewise, in the latter study increased uptake of Ca^{45} was observed during HK stimulation but total Ca remained unchanged. The authors implied that the increased uptake was related to tension development. However, Somlyo (1968) has questioned the validity of these results on the basis of the methodology used in these experiments. The inherent difficulties in differentiating small Ca movements against a large total background tissue Ca content were again reiterated by van Breemen (1970). He was unable to find any correlation between the tension development and increased Ca^{45} uptake in rabbit aorta in normal, Li and K solutions.

D. Calcium Pools in Excitation-Contraction Coupling in Smooth Muscle

The concentration gradient of Ca across the cell membrane is of the order of 10^4. It is assumed that the cytoplasmic Ca must be lower than 10^{-7}M in the relaxed muscle cell (Filo, Ruegg and Bohr, 1965) whilst the plasma concentration of free Ca is approximately 10^{-3}M (Walser, 1961). Therefore, the cell membrane has been assigned the function of a barrier against the influx of extracellular Ca down its concentration gradient, thus effectively compartmentalizing Ca into intra and extracellular pools. It is generally accepted that mechanical activity is brought about by an increase in cytoplasmic Ca to or beyond the threshold concentration for activation of the contractile proteins, whereas inhibition of mechanical activity is brought about by a lowering of cytoplasmic Ca.

In skeletal muscle, evidence supports the theory that to initiate contraction Ca ion is released from an intracellular structure, the sarcoplasmic reticulum. Relaxation occurs by rebinding of free intracellular Ca to this same structure (Weber, Herz and Reiss, 1963; Hasselbach, 1964; Martonosi, 1971). That the same structure performs the same function in smooth muscle has not been demonstrated. Indeed the sarcoplasmic reticulum is believed to be very meager compared to the highly organized reticulum of skeletal muscle. Nevertheless, in smooth muscle there is evidence that upon stimulation, particularly by drugs, intracellular bound Ca is released. In addition, there is evidence that an influx of extracellular Ca might occur on stimulation. This Ca influx has been observed upon depolarization

of the skeletal muscle membrane (Bianchi and Shanes, 1959) but it is insufficient to activate contractile machinery (Frank, 1971; Bianchi, 1968). This suggests that the Ca influx may be a trigger to release sarcoplasmic Ca.

The probability that Ca from both extracellular and intracellular stores was involved in excitation-contraction coupling in smooth muscle was hinted at by the observations that, in depolarized uterine muscle the effects of adding Ca or ACh to the incubation medium were independent and additive (Edman and Schild, 1961). This would suggest two Ca sites in this muscle or that Ach increases Ca conductivity. Further clarification of these uterine Ca pools was afforded by Daniel (1963). Based on studies of relaxation rates and rates of return of tension in Ca depleted muscle, he discounted simple diffusion of Ca as a means of raising or lowering internal free Ca. The same conclusion was reached in relaxation studies on the guinea pig ileum (Hurwitz et al., 1969). Daniel proposed that Ca was bound to the surface of the cell membrane and that stimulation with high K or Ach removed both Ca and its stabilizing effects on the membrane with a consequent influx of extracellular Ca through pores in the membrane. van Breemen and Daniel (1966) later showed that during the slow phase of Ca^{45} efflux from rat uterus HK but not Ach could displace a fraction of Ca^{45} supposedly from the membrane. Since no increase in uptake of Ca^{45} was observed with either HK or Ach, the idea of a net influx of extracellular Ca was rejected. It was therefore postulated that HK released Ca from a loosely bound membrane fraction which consequently released internal more tightly bound Ca. Ach, on the other hand, was only capable of

releasing the tightly bound intracellular Ca without releasing the loosely bound membrane fraction.

Evidence presented for the taenia coli suggests that as for uterine muscle, Ca is released from an intracellular binding site. This conclusion was drawn from the observation that HK, Ach and carbachol increased the rate of Ca^{45} efflux from this muscle with an apparent net loss of Ca (Schatzman, 1964). This loss would not be observed if a permeability increase to extracellular Ca had occurred to stimulation. Contrary to the results of Schatzman, Goodford (1964) observed no effect of these stimulants or metabolic inhibitors on the rate of Ca^{45} efflux. It was therefore suggested that increases in intracellular Ca were reduced by an active accumulation of microcrystalline Ca phosphate to a concentration above that of the extracellular Ca. During relaxation the deposit would dissociate independently of metabolic processes and diffuse down a concentration gradient to the exterior of the cell. The microcrystalline deposit in this muscle therefore might be considered in the same light as the sarcoplasmic reticulum in skeletal muscle.

In vascular smooth muscle evidence supports the postulate of two Ca pools in excitation-contraction-coupling. Bohr (1964) noted that the NA contracture in rabbit aorta was made up of an initial fast response followed by a slow tonic response. The fast response was much less affected by changes in external Ca than the slow response. It was inferred, therefore, that the two responses had different dependencies on external Ca. Hinke (1964) also demonstrated a complex relationship between external Ca concentration and the return of Na

contractions in Ca depleted rat tail artery. In contrast, the HK contraction bore a linear relationship to external Ca concentration. Based upon these observations and the observation that NA contractions could still be elicited at a time after HK contractures were abolished in Ca free medium, Hinke suggested that NA released a tightly bound Ca fraction which probably incurred an increased influx of extracellular Ca, whereas, HK increases the influx of extracellular Ca without releasing the tightly bound fraction. This model is a modification of Daniel's in that an influx of extracellular Ca is postulated.

Although there is a good deal of evidence to suggest the involvement of membrane bound Ca in excitation-contraction coupling in vascular and other smooth muscle, Somlyo in a recent review (Somlyo and Somlyo, 1968) considers the current evidence does not rule out the possibility that drugs increase the Ca permeability above that of HK.

Although these various Ca pools have been proposed the site of binding of intracellular Ca, involved in activation of contractile proteins, remains obscure. As stated previously the sarcoplasmic reticulum is believed to be poorly developed in smooth muscle yet other structures have been observed that might well serve as Ca sinks. The existence of a longitudinal smooth sarcotubular system has been observed in intestinal muscle (Lane, 1967; Gabello, 1971). Furthermore, ATPase activity has been demonstrated in the tubular system of mouse bladder (Lane, 1967) implying the presence of an ion transport system. In skeletal muscle ATPase activity is also found in the sarcotubular system. Associated with the plasma membrane of smooth muscle are numerous vesicles and although their function is unknown it has been

suggested that they may be associated with relaxing factor because of their close association with mitochondria and endoplasmic reticulum (Nagasawa and Suzuki, 1967; Gabello, 1971).

E. Introduction to the Problem

In 1969, van Breemen introduced the lanthanum (La) ion as a tool in the study of Ca movements in vascular smooth muscle cells, incurred by stimulatory agents. Lettvin, Pickard, McColloch and Pitts (1964) had predicted that lanthanum would have a greater affinity for Ca binding sites than Ca itself. It was demonstrated that Ca movement across a cholesterol phospholipid artificial membrane was pH-dependent, suggestive of Ca transport mediated by fixed negative sites within the membrane (van Breemen and van Breemen, 1969). Furthermore, the rate of transport of Ca^{45} across the membrane could be increased by addition of Ca to the side of the membrane from which Ca^{45} was emerging. La when added to both sides of the membrane inhibited the Ca flux. Furthermore, van Breemen and DeWeer (1970) showed that efflux of injected Ca^{45} from squid axon was inhibited 85% by La.

Based upon the two assumptions that La blocks influx and efflux of Ca across the smooth muscle cell membrane, the effects of La were tested on the HK and NA contractions in rabbit aortae with the results that La could abolish the phasic part of the HK contracture but not the NA contracture when added during the tonic part of the individual responses. From these results, it was suggested that the HK phasic response was supported by extracellular Ca; the NA phasic response was supported by intracellular release of Ca while the HK tonic response was supported by intracellular Ca and the tonic NA response by extracellular Ca (van Breemen, 1969).

Further studies with La in the wash medium have shown its capability of reducing the tissue Ca^{45} content of rabbit aorta compared to controls washed in Ca free medium (van Breemen et al., 1970). It has therefore been assumed that La is capable of displacing extracellular bound Ca, leaving intracellular Ca relatively unaffected. This contention was further supported by the finding that good correlation could be achieved between Li and HK induced tension development and Ca^{45} content in this muscle after La treatment (van Breemen et al., 1970). As mentioned in a previous section there was no correlation in the absence of La.

The present study is designed, first to test the basic assumptions underlying the use of La, i.e., a) that La displaces extracellular Ca; b) that La blocks efflux of intracellular Ca; and c) that La blocks influx of Ca into the cell and secondly, to use La in the study of Ca pools involved in excitation-contraction coupling in rabbit aortae.

II. MATERIALS AND METHODS

A. Materials

1. Solutions

Tris buffered Krebs (NKR) of the following composition was used: NaCl, 115mM; KCl, 4.6mM; $CaCl_2$, 2.47mM; $MgSO_4$, 1.2mM; tris-(hydroxymethyl)aminomethane (Tris), 21.9mM; glucose, 50mM. The Tris buffer was adjusted to pH 7.4 with 12N HCl. The Krebs Solution was bubbled with 100% O_2 and periodic checks of the pH showed that it did not change during experiments.

Isotonic High Potassium Krebs (HKR) was attained by substituting KCl for NaCl yielding a final K^+ concentration of 119.6mM.

Calcium free (Ca-free) solutions were obtained by deleting CaCl.

2. Drugs and Stimulants

The stimulants used were Noradrenaline Bitartrate (NA) - (Nutritional Biochemicals) and high potassium ion concentrations (HK) administered in the form of HKR. Lanthanum (La, La^{+++}) chloride was obtained from Fisher Chemical Co.

3. Isotopes and Liquid Scintillation Fluid

The isotope of calcium used was Ca^{45} obtained from Amersham Searle as the chloride.

The scintillation fluid used to measure β-emission from Ca^{45} was devised by Bray (1960) and contained 60g naphthalene, 4g PPO (2,5-diphenyloxazole), 0.2g POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene], 100 mls methanol and 20 mls ethylene glycol dissolved in 1 litre dioxane.

B. Methods

1. Tissue Preparation

Young New Zealand rabbits, weighing 3-4 pounds were killed by a blow on the head. The chest cage was cut open and the aorta removed between the heart and diaphragm. The vessel was immediately placed in Tris-buffered ringer to wash out blood; thereafter it was placed in a petri dish containing Tris ringer for removal of fatty connective tissue. The aorta was then cut into eight to ten rings of approximately three to four millimeter lengths.

2. Flux Studies

a) Efflux experiments

Aortic rings were incubated for 1 hour at 37°C in NKR. They were then transfered to Ca^{45} labelled NKR (specific activity 10^6cpm/μMole Ca for 1 hour followed by continued uptake in either Ca-45 labelled NKR, HKR or NKR + NA 10^{-6}g/ml (10^6cpm/μMole Ca in each case) for 1 hour. The tissues were then rinsed for 5 seconds in 100 mls of wash medium and effluxed into 4 mls of various Ca-free media at 5 minute intervals for the desired length of time. At the termination of of an efflux study, tissue rings were cut open, blotted lightly 3 times between tissue paper and weighed. Tissue Ca^{45} determinations were then made. The 4 mls effluent samples, contained in mini vials (Nuclear Associates), were evaporated to dryness at 40-50°C and redissolved in 0.2 ml double distilled water. Five mls of scintillation fluid was then added and the samples counted after a period of time allowed for dark adaptation and cooling.

Further desaturation studies were conducted in which the tissue Ca^{45} content was determined rather than the Ca^{45} contained in the effluent. These tissues were incubated in Ca^{45} labelled media, as described above, then rinsed for 5 seconds in 100 mls of wash media followed by continued washing for specific lengths of time in a fresh volume (250 mls) of the same wash media used for rinsing. Control and experimental tissues were washed separately with usually 4 tissues per 250 mls of wash medium. After washing, the residual tissue Ca^{45} was determined.

In attempting to measure the effect of La on intracellular Ca^{45} by the above procedure, control and experimental tissues were washed in identical non-radioactive solutions except for the absence or presence of La. In experimental tissues, La was present for the duration of each time period. However, controls were also exposed to La during the last 20 minutes of each time period. This was done in order to eliminate any extracellular bound Ca that might have obscured differences in intracellular Ca^{45} content between control and experimental tissues.

b) Uptake experiments

Aortic rings were allowed to recover for 1 hour in NKR at 37°C. The rings were then incubated 1 hour in Ca^{45} labelled NKR (10^6cpm/μMole Ca) followed by 1 hour incubation in Ca^{45} labelled NKR, HKR or NKR + NA 10^{-6} g/ml (10^6cpm/μMole Ca in each case). One group of tissues was exposed to Ca^{45} labelled NKR whereas the experimental tissues were exposed to Ca^{45} labelled NKR for 115 minutes followed by 5 minutes in Ca^{45} labelled HKR. Thus both control and experimental tissues were exposed to the isotope for the same length of time (2 hours).

After incubation was complete, tissues were rinsed for 5 seconds in La containing Ca-free NKR followed by a 1 or 3 hour wash in an identical medium. Tissues were then blotted, weighed and the Ca^{45} content determined.

c) Determination of significant differences

The unpaired t-test was used for these determinations of significance.

3. Tension Studies

Aortic rings isolated as described earlier were suspended between two stainless steel hooks. The upper hooks were attached to Beckman isometric strain gauges while the lower hooks were attached to the bath aerators. The 20 ml capacity baths were maintained at 37°C and bubbled with oxygen. Each loop was allowed to recover under 5 gms tension for 2 hours in NKR. Loss of tension during the 2 hour recovery period was corrected before experiments were begun. Bathing media was changed with 20 ml pipettes and additions of NA and La were made by injection from concentrated stock solutions to bring the bath to the desired concentration. Preliminary dose response relationships showed that approximately 4×10^{-7} g/ml NA stimulated a maximal response but the presence of La necessitated an increase in NA concentration to 4×10^{-6} g/ml to elicit a maximal response; therefore 10^{-6} g/ml NA was used in the absence and, in most cases, 4×10^{-6} g/ml NA in the presence of La.

4. Procedure for Determination of Tissue Calcium Content

The determination of tissue Ca content was made on the assumption

that there was homogeneity of distribution of isotopes in the incubation medium and the tissue.

After experimental procedures had been conducted on muscle rings, they were cut open, blotted three times between tissue paper, weighed and digested overnight with 1 drop of water and 0.25 ml of NCS (Nuclear Chicago) - a tissue solubilizer. Digestion was done in mini-vials at a temperature of about 40°C. To the digest was added 5 ml of Bray's scintillation fluid. Each sample was then dark-adapted and cooled to 4°C and counted in a Picker Nuclear Three Chanel Liquimat.

The uptake media was diluted 1:100 with DDH_2O. One ml of this solution was counted in 5 mls of Brays scintillation fluid while total Ca was measured, as described below, from a sample of the remaining solution. The specific activity (cpm/μMole Ca) of the uptake media was then circulated.

The tissue Ca^{45} content was calculated by dividing tissue cpm/g by the specific activity of the media to give mMoles Ca^{45}/kg wet weight.

Quench corrections were made where necessary using the channels ratio method. For total Ca content, incubation media was diluted 1:100 and the absorption measured at a wavelength of 422 mμ on an SP90 Unicam flame spectrophotometer. The calcium concentration was then determined by comparison to a standard curve.

For total tissue Ca aortic rings were blotted, weighed, digested in concentrated HNO_3 and evaporated to dryness. The resultant salt was dissolved in 5 mls 0.1N HCl containing 0.5% LaCl. The samples were then read on the flame spectrophotometer and the Ca content

determined by comparison to a standard curve derived from standards containing 0.5% LaCl.

III. RESULTS

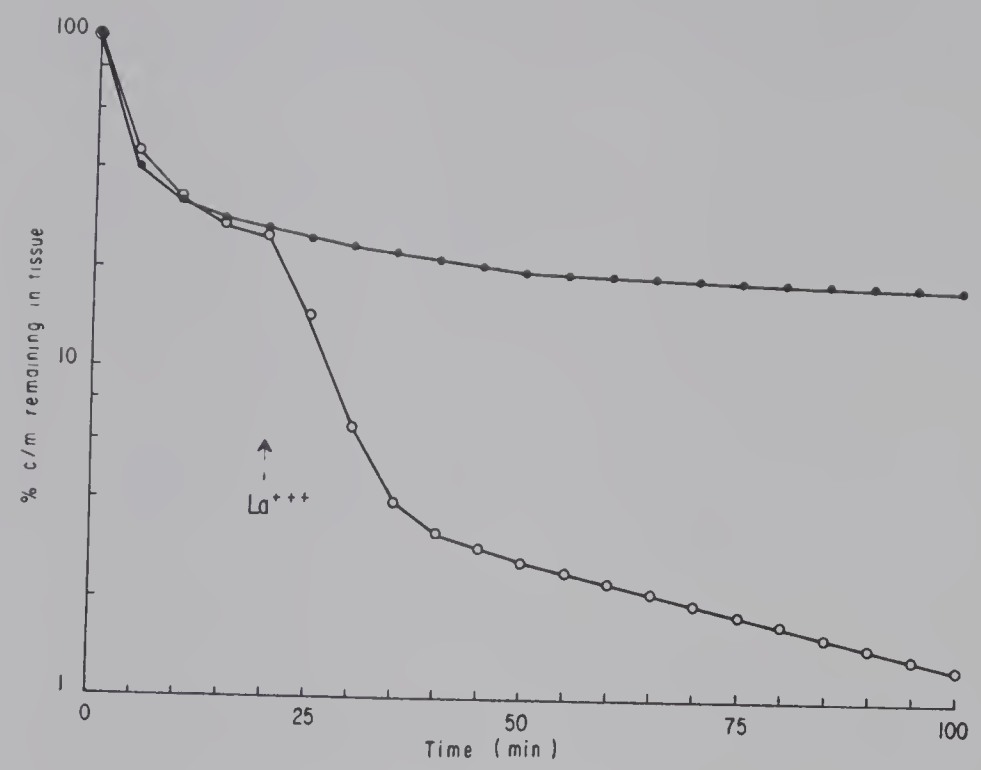
A. Calcium Content of Rabbit Aorta

The Ca content of rabbit aortic rings incubated 1 hour in NKR followed by 2 hours in Ca^{45} labelled NKR at 37°C was 3.0430 ± 0.0430 mMoles/kg wet weight (n=4)(Ca^{45} method). Similarly the total Ca content determined after 3 hours in NKR at 37°C was 3.0701 ± 0.0785 mMoles/kg wet weight (n=5)(Atomic absorption method). Both values were obtained from the same experiment. The mean Ca^{45} value derived from labelled NKR incubated tissues taken from a variety of different experiments in this presentation was 3.3492 ± 0.0553 mMoles/kg wet weight (n=19).

B. Effect of La on the Loss of Ca^{45} from Rabbit Aortic Rings

The ability of La to displace tissue Ca, presumably by virtue of its stronger affinity, from negative binding sites was measured by observing the changes in rate of loss of Ca^{45} from aortic rings exposed to La. Tissues were loaded in Ca^{45} labelled NKR, HKR and NKR + NA 10^{-6} g/ml solutions as described in Methods (section 2) and then the efflux of Ca^{45} into Ca-free or Ca-free EGTA (0.5mM) NKR was followed. La (2mM) was added at 20 or 40 minutes after beginning the efflux from experimental tissues and was omitted in controls. La remained in the wash solution for the balance of the 100 minute experiments. The times of La addition were arbitrarily chosen but both fell during the slow phase of desaturation presumably after free extracellular Ca^{45} had been lost. Control curves were analysed by the method of Cook and Taylor (1971) but revealed inconsistent values for the number of compartments, bound fractions, and half-times of compartmental desaturation. Four paired curves were constructed for each group of tissues loaded in Ca^{45} labelled NKR, HKR and NKR + NA 10^{-6} g/ml. Typical results are shown in Figure 1 (a,b,c,d), Figure 2 (a,b,c,d) and Figure 3 (a,b,c,d). La increased the rate of loss of Ca^{45} into Ca-free NKR after both times (20 and 40 minutes) of La addition (Figures 1, a and c; 2, a and c; 3, a and c). This increase in Ca^{45} efflux was initially very rapid but slowed to control levels after 20-25 minutes. The qualitative changes brought about by La addition to tissues loaded in stimulant and normal solutions were similar; thus representative

a



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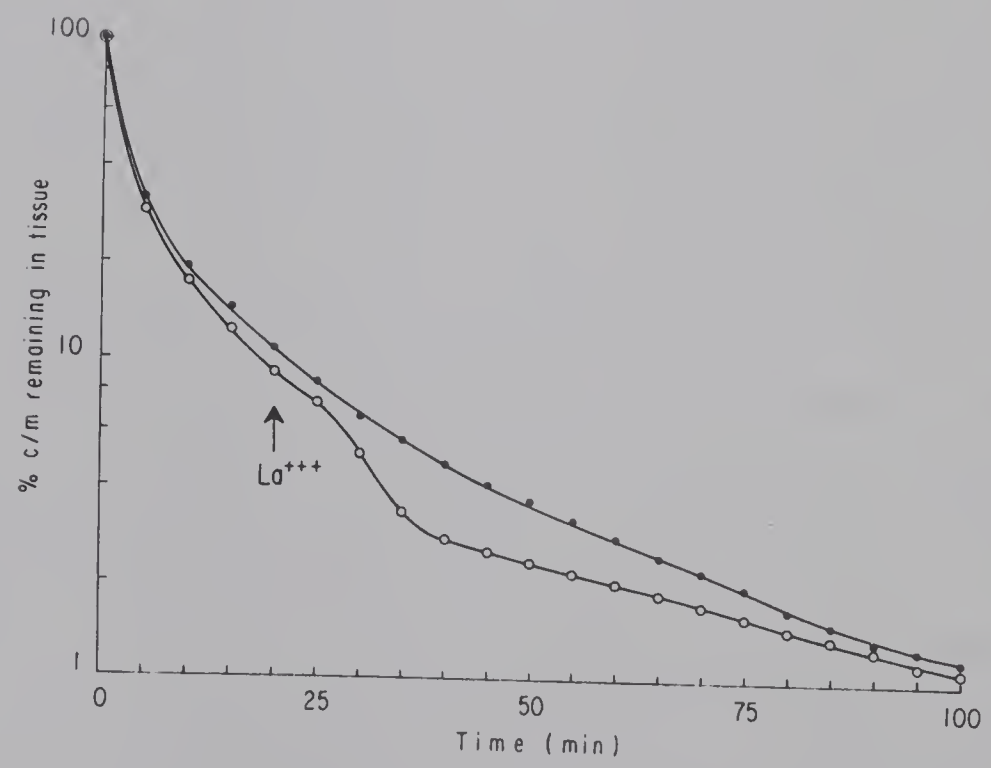
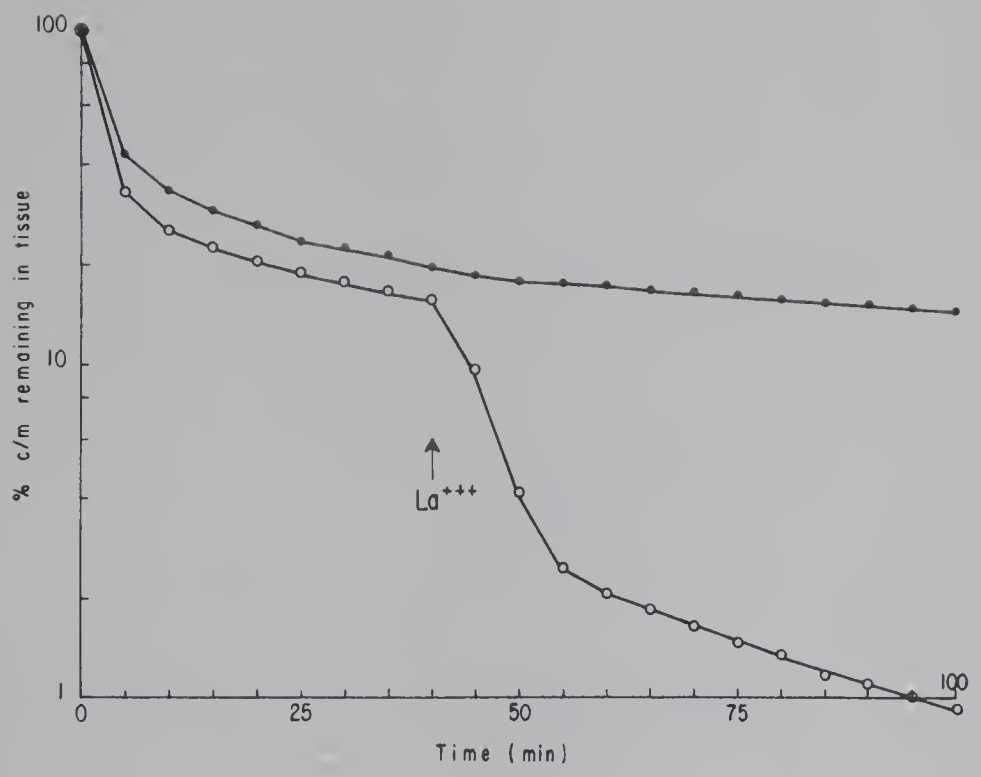


Figure 1, a and b. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in NKR (Ca^{45}).

Tissues in (a) and (b) were incubated in NKR (Ca^{45}) for 2 hours. Washout solutions were Ca-free NKR without (a) or with 0.5mM EGTA (b). La 2mM was added to experimental tissues (o) after 20 minutes. Control (●).

c



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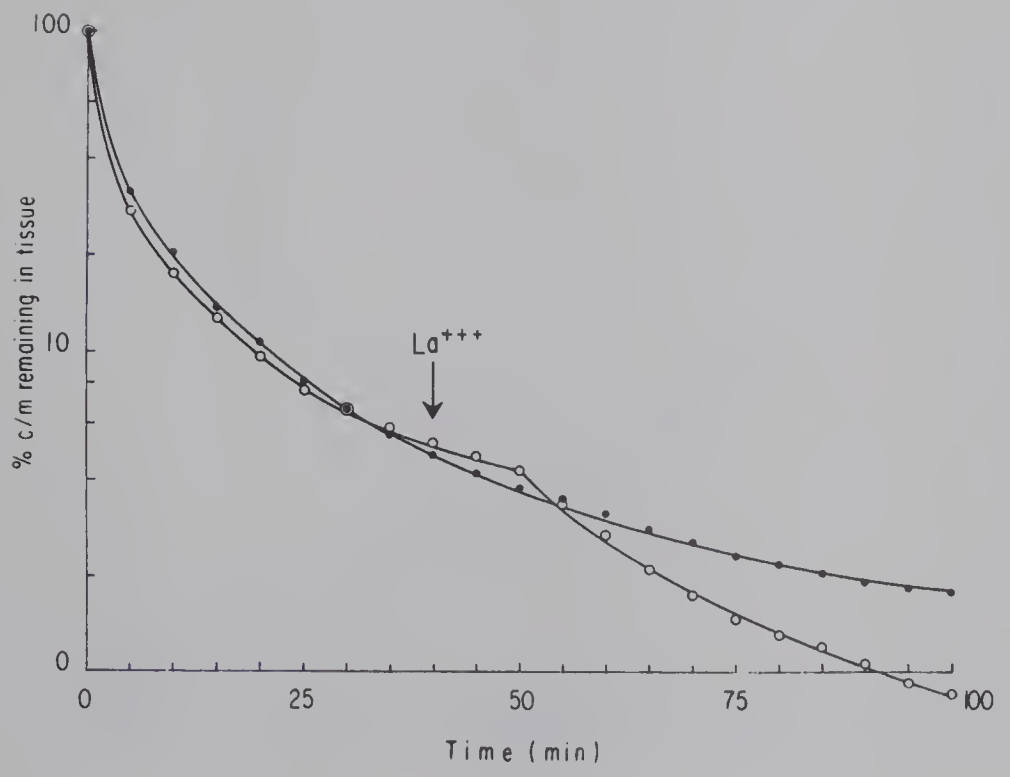
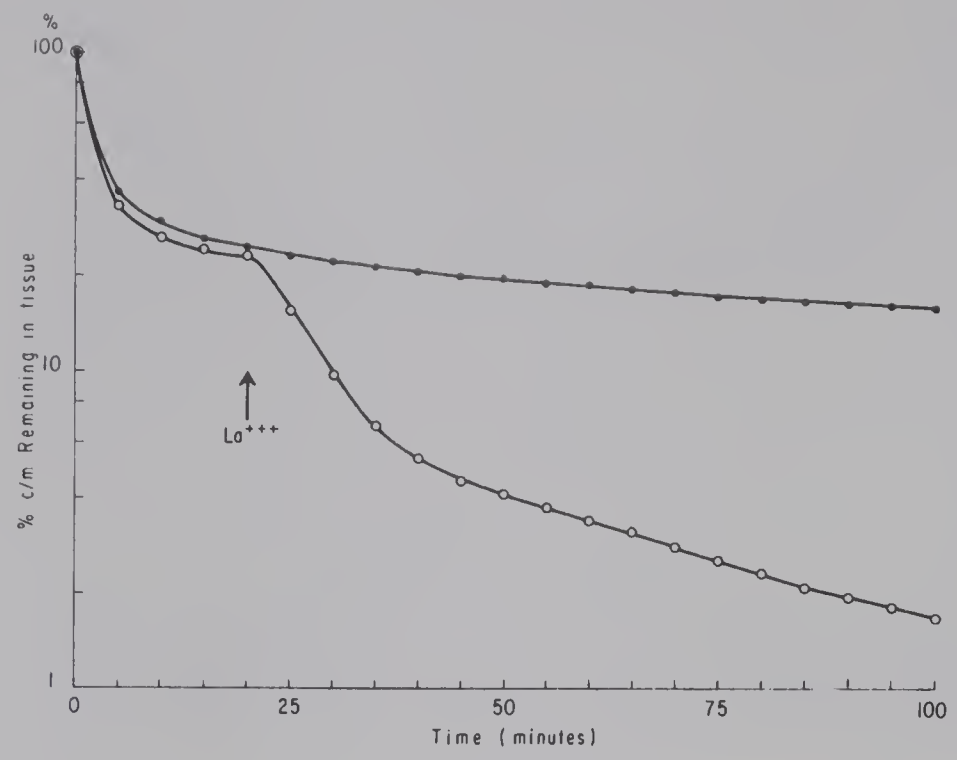


Figure 1, c and d. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in NKR (Ca^{45}).

These tissues were treated as described in Figure 1, a and b, but La was added after 40 minutes.

a



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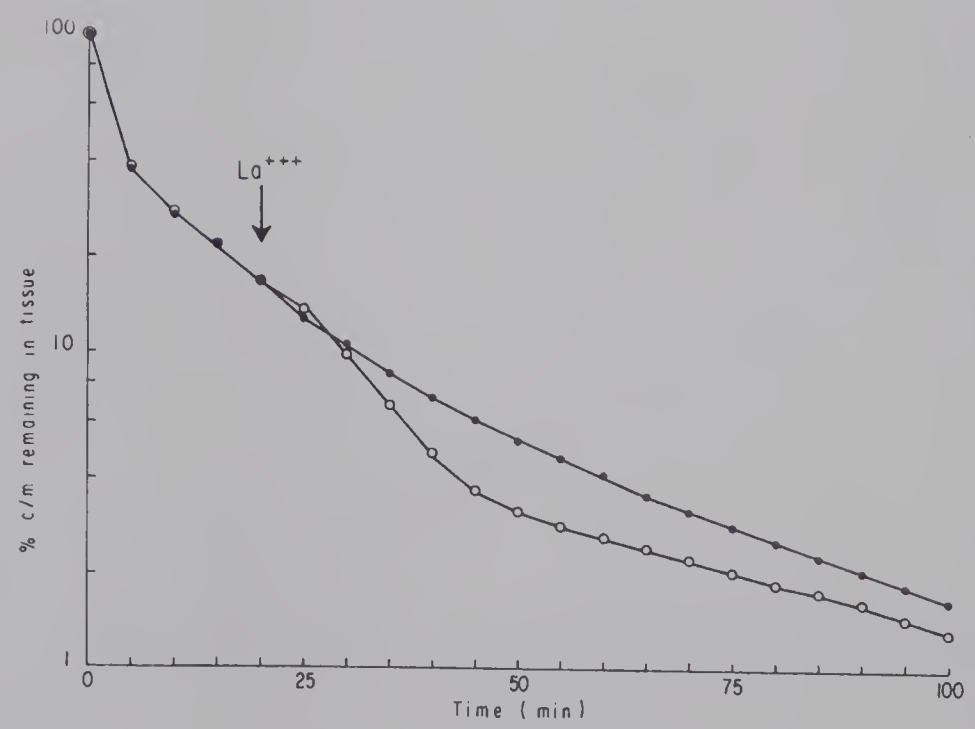
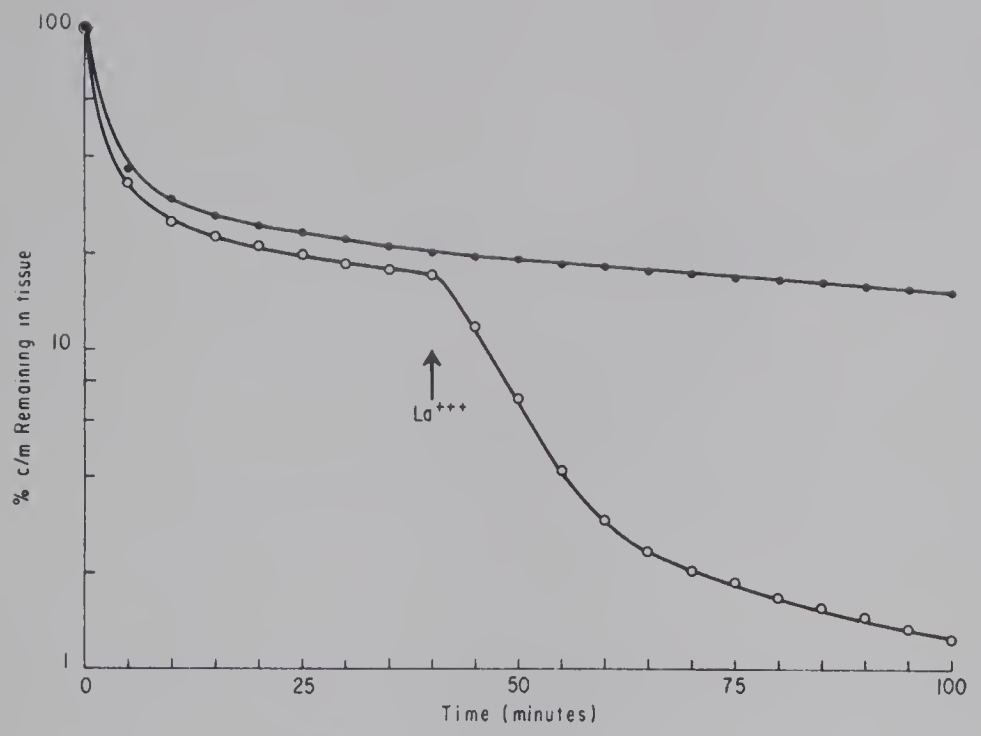


Figure 2, a and b. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in NKR(Ca^{45})+NA.

These tissues were incubated 1 hour in NKR-Ca^{45}, and 1 hour in NKR-Ca^{45} + NA 10^{-6}g/ml, then washed as described in Figure 1, a and b.

c



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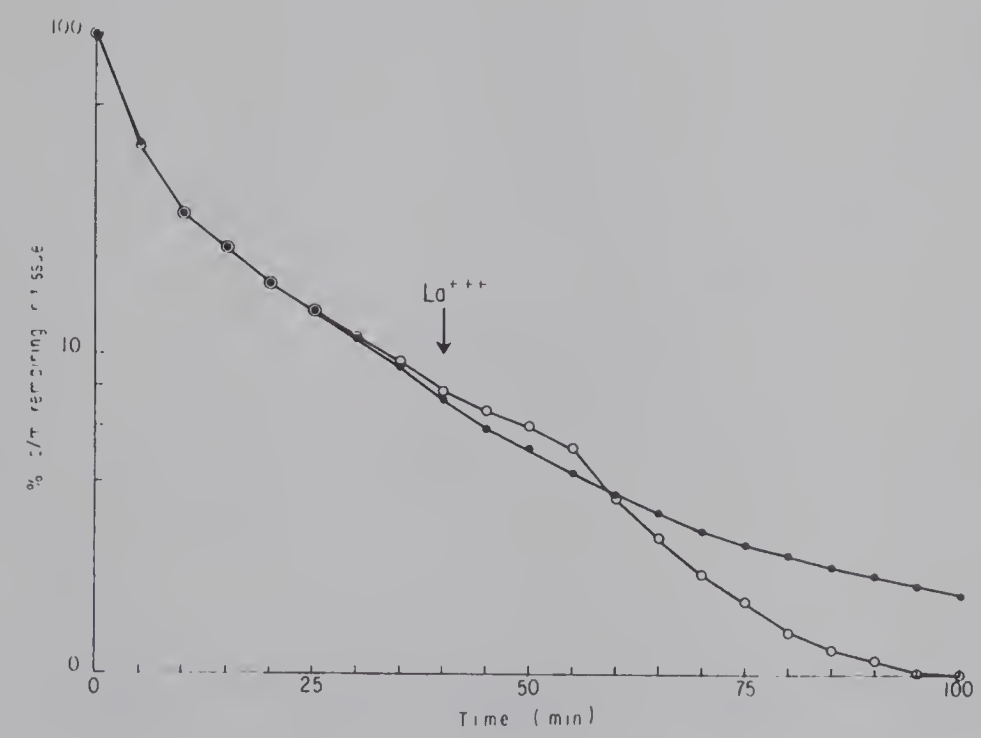
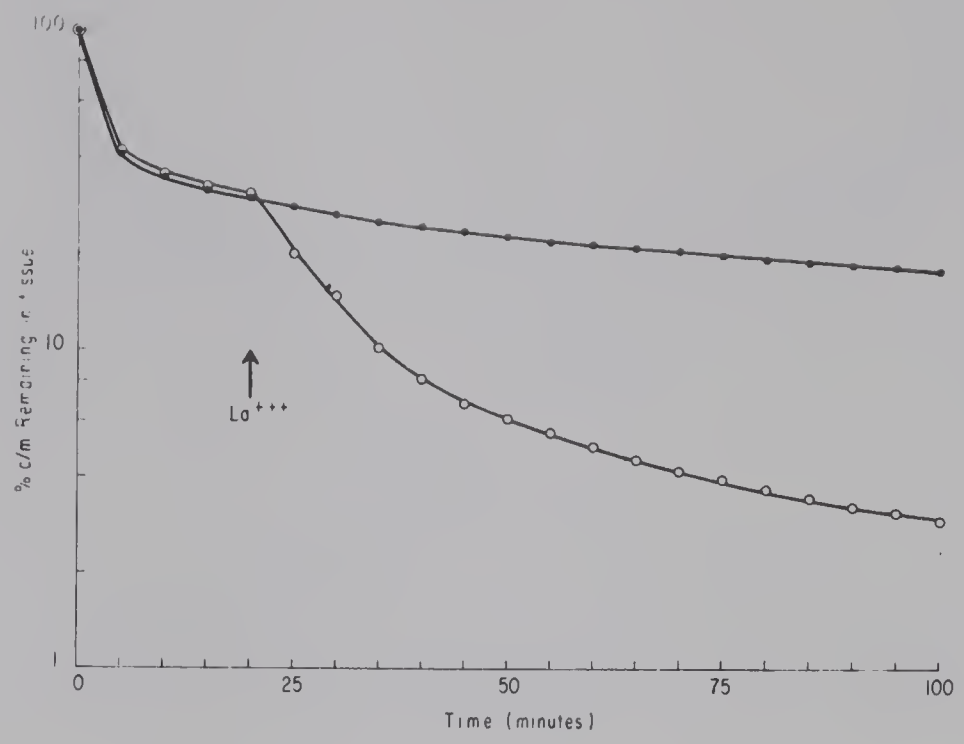


Figure 2, c and d. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in NKR (Ca^{45}) + NA.

These tissues were treated as described in Figure 2, a and b but La was added after 40 minutes.

a



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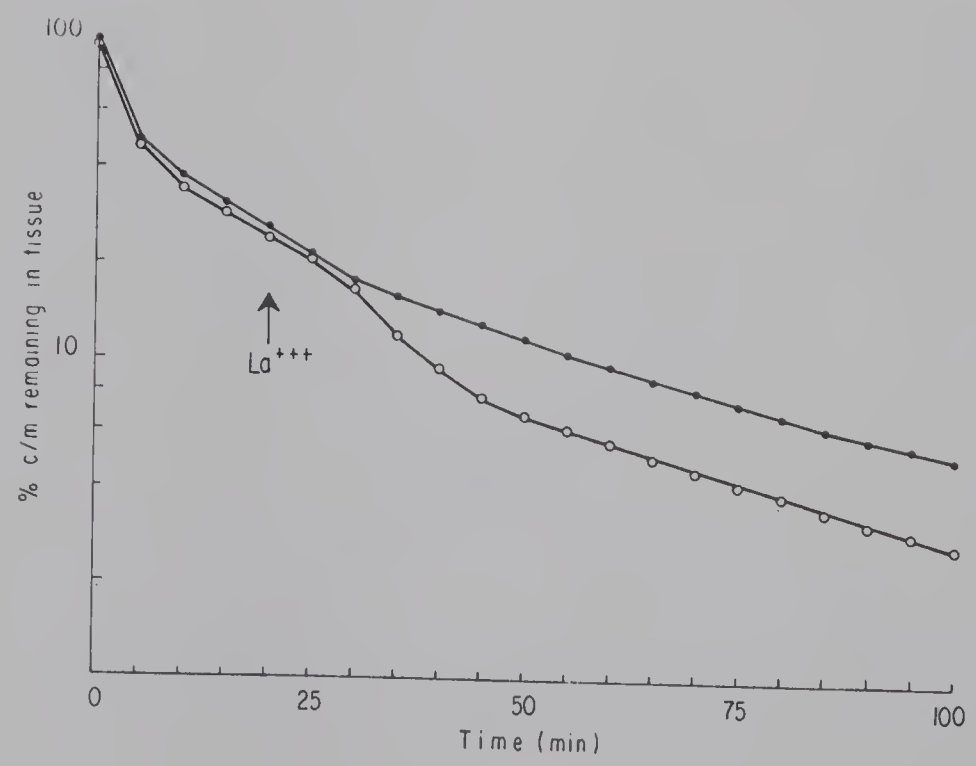
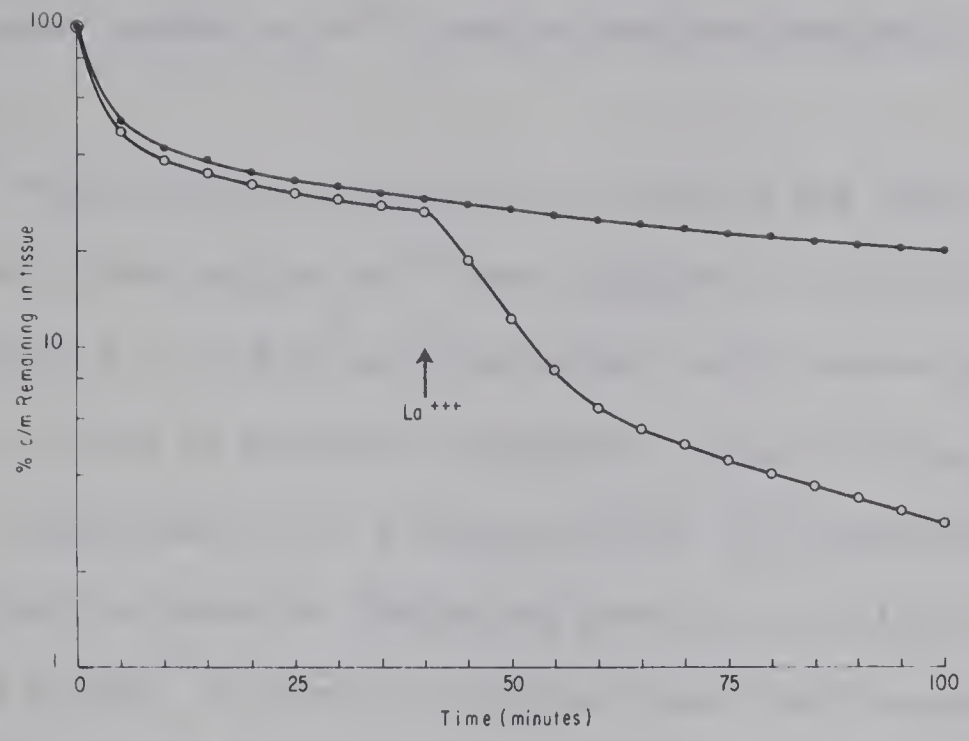


Figure 3, a and b. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in HKR(Ca^{45}).

These tissues were incubated 1 hour in NKR Ca^{45} and 1 hour in HKR-Ca^{45} then washed as described in Figure 1, a and b.

c



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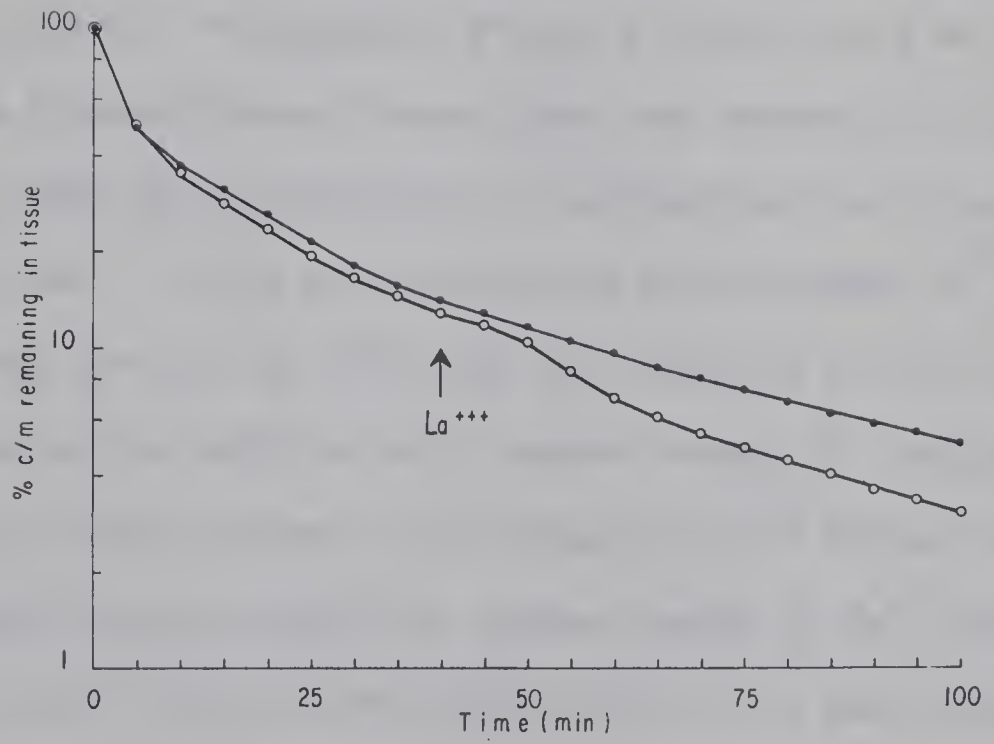


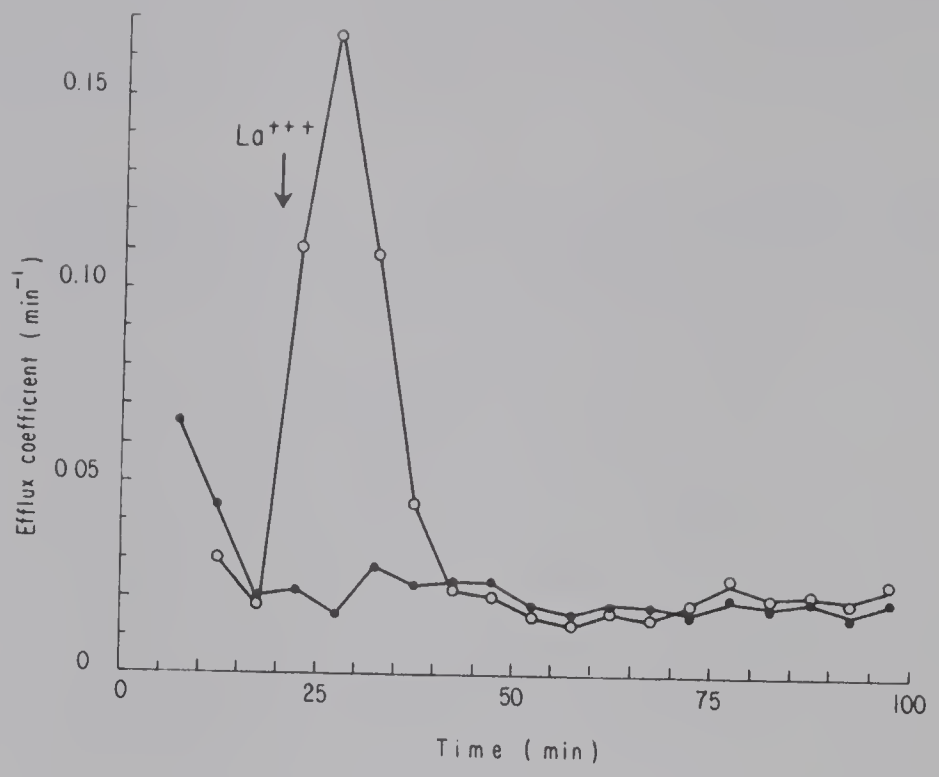
Figure 3, c and d. The effect of La on the rate of Ca^{45} efflux from rabbit aorta incubated in HKR(Ca^{45}).

These tissues were treated as described in Figure 3, a and b, but La was added after 40 minutes.

efflux coefficient curves constructed from data obtained from the tissues loaded in Ca^{45} labelled NKR are presented in Figures 4, a and b.

When EGTA was present in the Ca-free NKR there was only a small increase in the rate of Ca^{45} loss (Figures 1, b and d; 2, b and d; 3, b and d; 4, c and d) and this effect had a latent period of 10-15 minutes. Since La appeared to displace some Ca^{45} inaccessible to EGTA and its displacement had a latent period, the possibility was entertained that La might be displacing bound Ca, possibly that normally released by NA. It has been proposed that this NA-releasable Ca is located inside the vascular smooth muscle cells (Hinke, 1965; van Breemen, 1969). Furthermore, Figure 5 shows that a NA response can still be attained after tissues have been exposed for 20 minutes to Ca-free, EGTA NKR particularly in unstimulated (n=4) and HK stimulated tissues (n=4). If La were displacing NA-releasable Ca^{45} thereby increasing the rate of Ca^{45} loss then addition of NA to the efflux media before the addition of La should remove Ca from its binding sites and thereby prevent the increase in Ca^{45} efflux caused by La. This prediction was tested in tissues loaded in Ca^{45} labelled NKR and effluxed into either Ca-free (EGTA) NKR or the same solution containing 10^{-6} g/ml NA. La was added after 20 minutes of effluxing to both 4 experimental and 4 control tissues and remained in the wash solution for the balance of the 60 minute experiment. The desaturation and efflux coefficient curves are presented in Figure 6 (a and b) and show that La increases the rate of Ca^{45} loss similarly in the presence or absence of NA.

a



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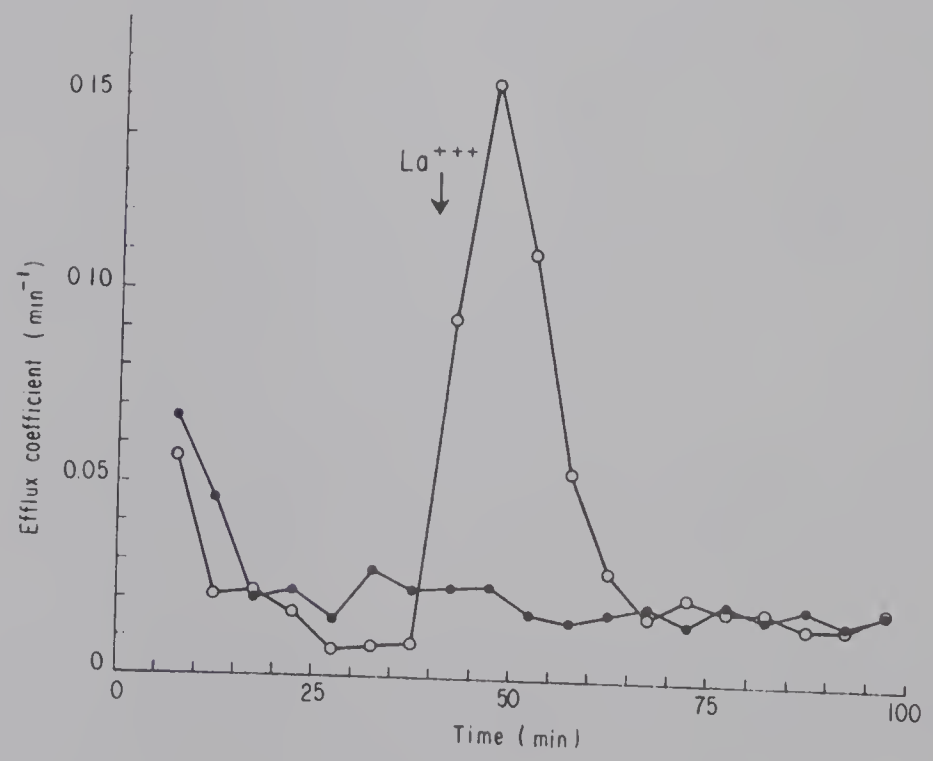
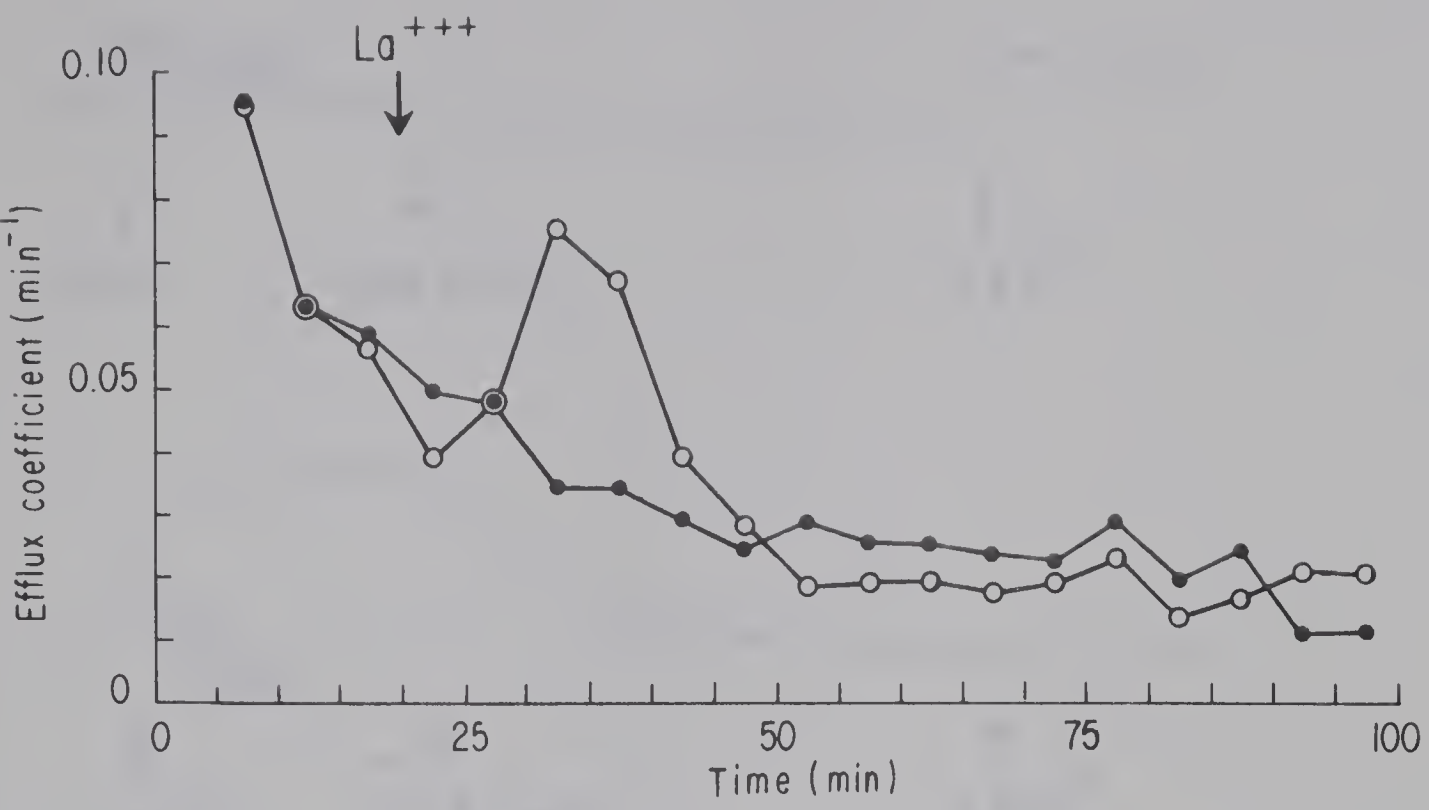


Figure 4, a and b. Effect of La on the Ca^{45} efflux coefficient.

The above figures are derived from data obtained from Ca^{45} efflux data from which figure 1, a and c was constructed. La was added after 20 (a) or 40 minutes (b). The efflux coefficients were calculated by dividing the efflux rate (cpm/min) during a period by the cpm remaining in the tissue at the end of that period.

c



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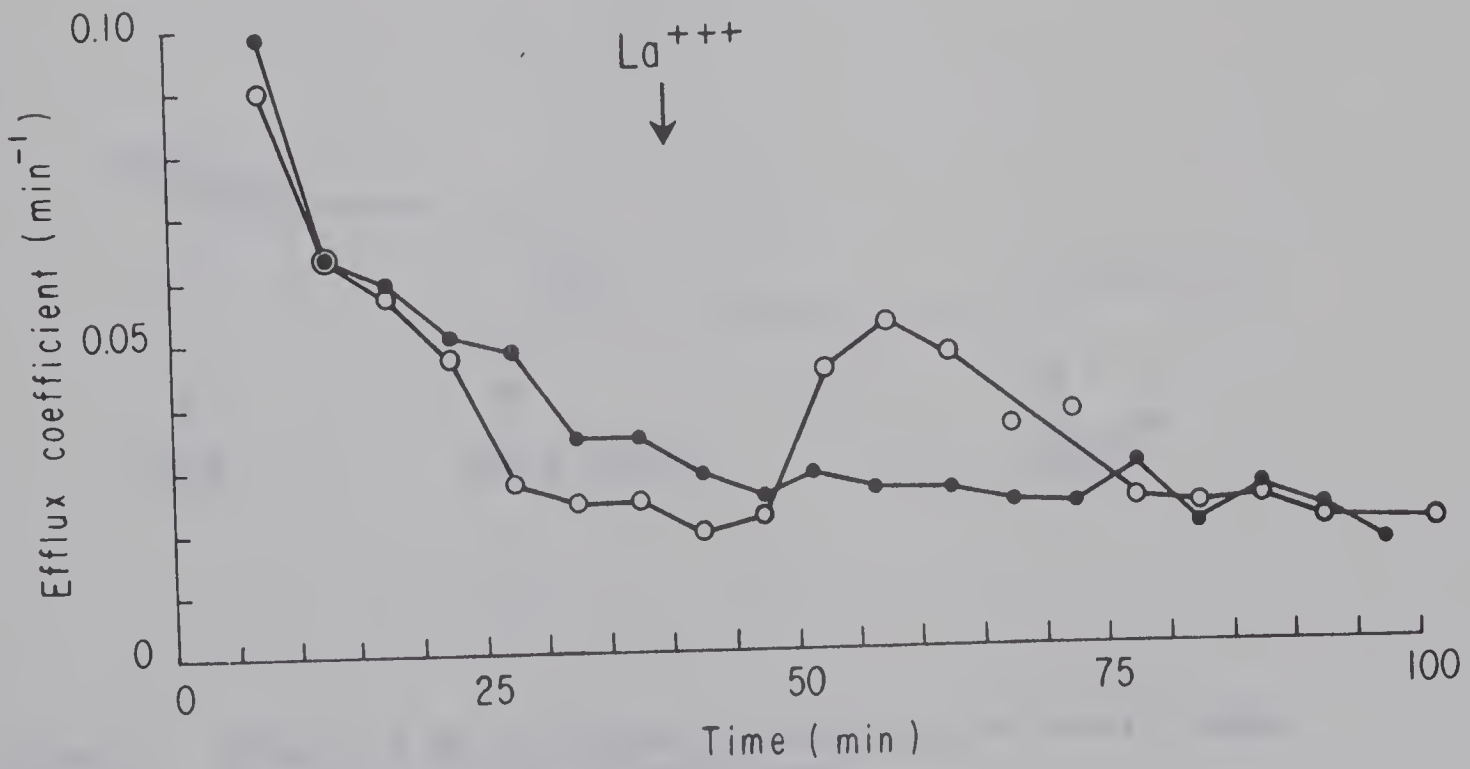
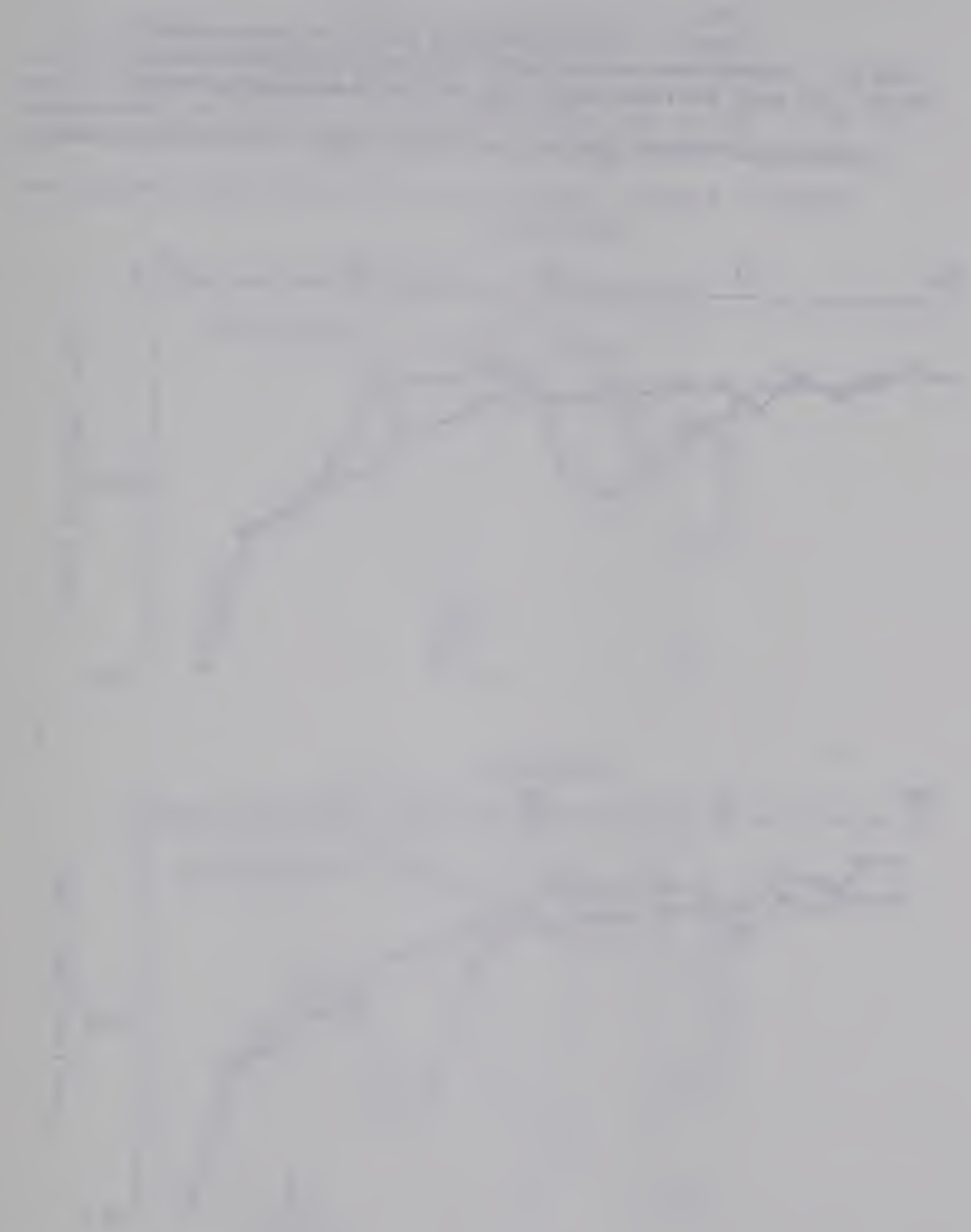


Figure 4, c and d. Effect of La on the Ca^{45} efflux coefficient.

These figures are derived from data obtained from Ca^{45} efflux data from which Figure 1, b and d were constructed. La was added after 20 (c) or 40 minutes (d).



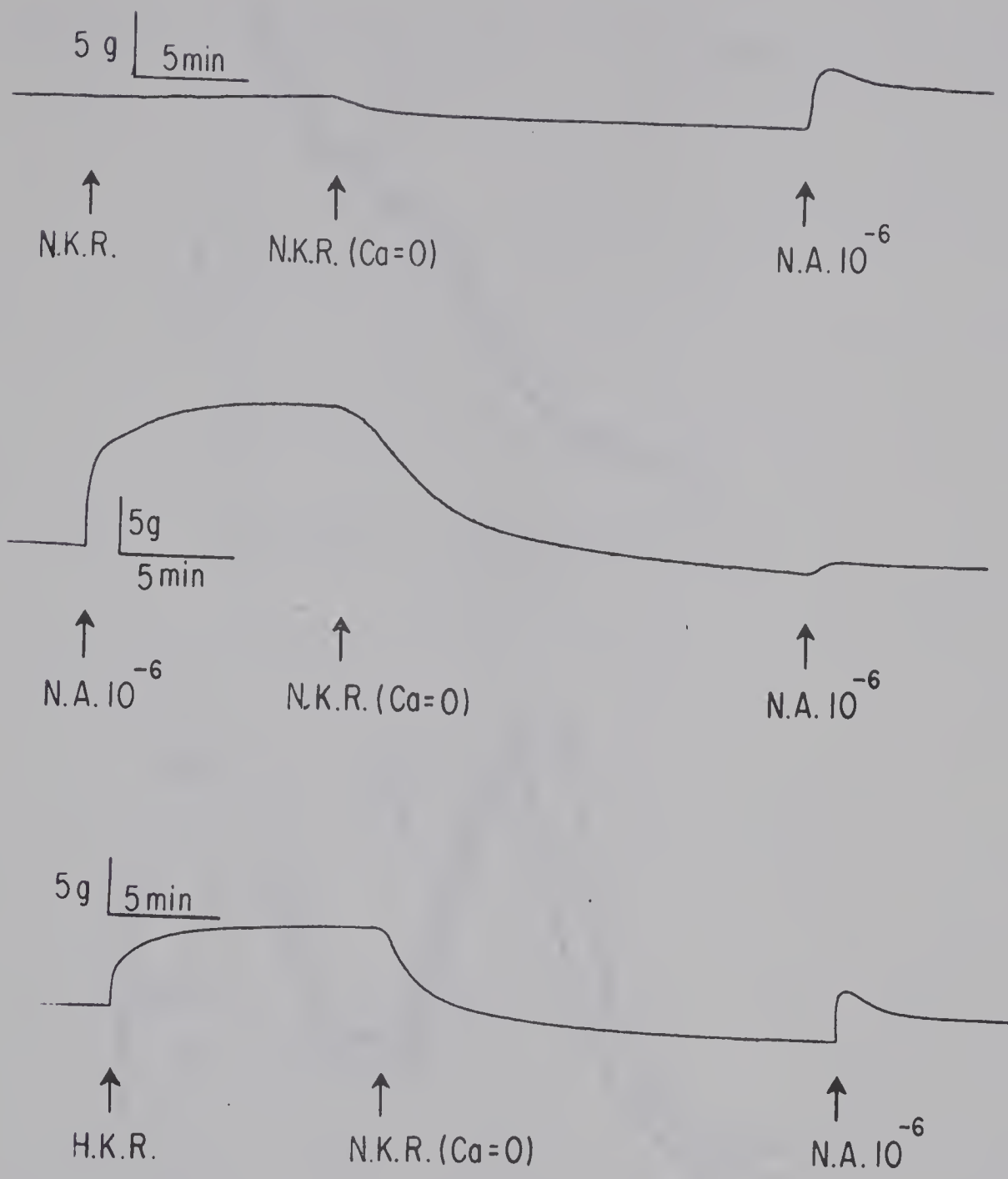
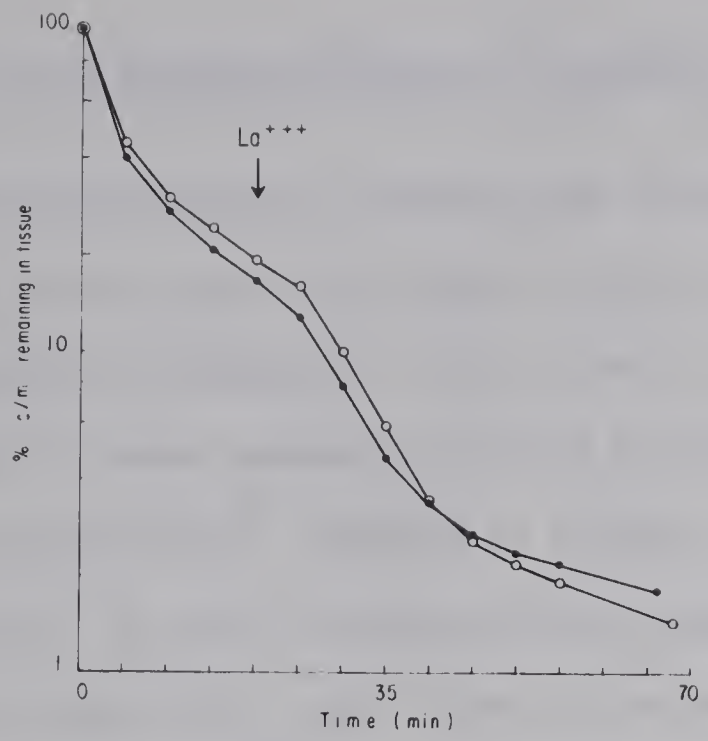


Figure 5. Effect of NA (10^{-6}g/ml) stimulation of aortic rings relaxed in Ca-free (EGTA) NKR.

Three tissues from the same animal were treated and their response to NA compared.



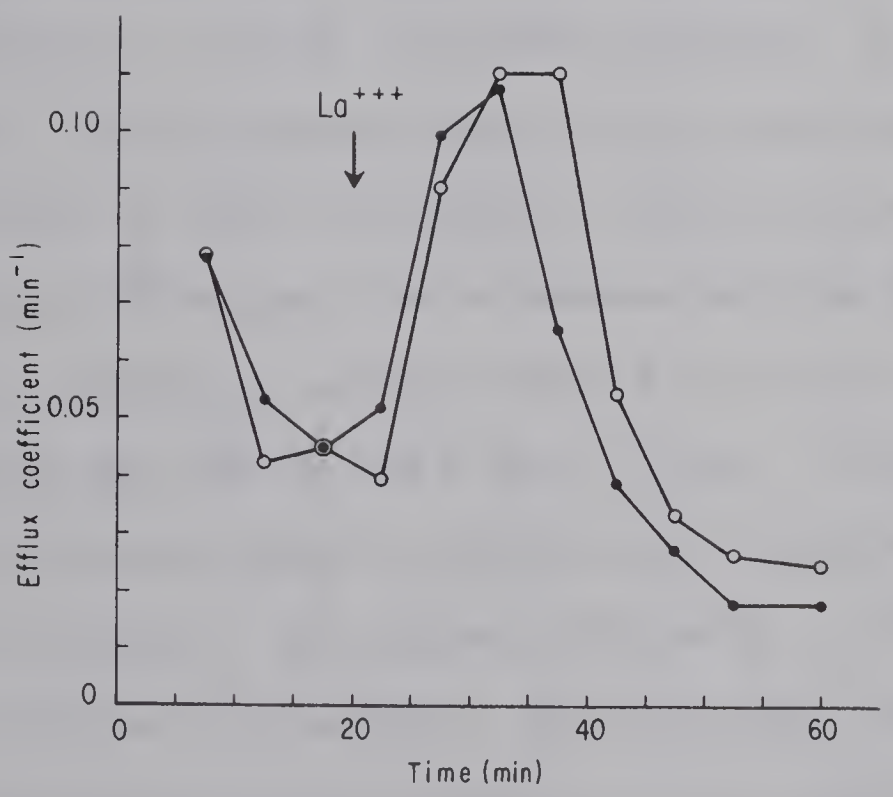


Figure 6. Effect of La on the rate of Ca^{45} efflux in the presence of NA

Tissues were incubated in NKR-Ca^{45} and washed out into Ca-free (EGTA) NKR with (o) or without NA 10^{-6}g/ml (●). La (2mM) was added to both tissues after 20 minutes. Both figures were constructed from data obtained from one typical pair of tissues. The efflux coefficients were calculated by dividing the efflux rate (cpm/min) during a period by the cpm remaining in the tissue at the end of that period.

C. Effect of La on Residual Tissue Ca^{45} Content of Rabbit Aortic Rings

If the assumptions are correct that La both displaces extracellular bound Ca and blocks the efflux of intracellular Ca (van Breemen, 1969; van Breemen and McNaughton, 1970), then it would be expected that Ca^{45} loaded tissues washed in Ca-free La solutions, would contain elevated intracellular Ca^{45} compared to tissues washed in an identical La-free solution. To test this expectation, groups of tissues labelled with Ca^{45} were washed for 1 and 3 hours in La-containing Ca-free NKR and their residual Ca^{45} content compared to those of control tissues washed in a similar but La-free solution. In these experiments the control tissues although termed 'La-free' were washed for the last 20 minutes of each wash period in 2mM La solution to remove extracellular Ca^{45} as described in Methods (section 2a). The results of these experiments are shown in Table I for tissues loaded in Ca^{45} labelled NKR, HKR and NKR + NA 10^{-6} g/ml. In each case the experimental tissues exhibit a significantly increased Ca^{45} content compared to controls. (The effect of NA and HK on Ca^{45} uptake is covered in section D of Results). This data when considered in light of observations from desaturation curves (Figures 1, 2, 3) appear at first glance to be contradictory. The desaturation data suggests that La decreases tissue Ca content whereas data contained in Table I shows La to have the opposite effect. The two contradictory observations can be reconciled by predicting that a point in time is reached during the wash in the above experiment where the percentage Ca content of the experimental and control tissues is identical. Before this point

TABLE I

Effect of La on the efflux of Ca^{45} (mmoles/kg) †

Uptake Medium	Wash Medium	Ca^{45} content after La (2mM) wash for 1 hr	n	3 hrs	n
NKR	Ca-free NKR (control)	0.0947 ± .0041	4	0.0211 ± .0012	4
	Ca-free NKR + LA	0.1585 ± .0029	4	0.0475 ± .0022	4
NKR + NA 10^{-6} g/ml	Ca-free NKR (control)	0.1009 ± .0072	4	0.0407 ± .0029	4
	Ca-free NKR + La	0.1524 ± .0064	4	0.0693 ± .0009	4
HKR	Ca-free NKR (control)	0.1545 ± .0043	4	0.0404 ± .0012	4
	Ca-free NKR + La	0.2151 ± .0045	4	0.0732 ± .0019	4

† one of two experiments.

$p<.05$ in all cases comparing ± La.

the experimental tissues contain less Ca^{45} than controls whereas after this point experimental tissues contain more than the controls. To test this prediction Ca^{45} desaturation curves were constructed from data obtained by following identical wash procedures as those in the above experiment. One of 4 similar paired curves is shown in Figure 7 and shows the predicted crossover of Ca^{45} desaturation curves does occur at a time 40-60 minutes after the initiation of washing.

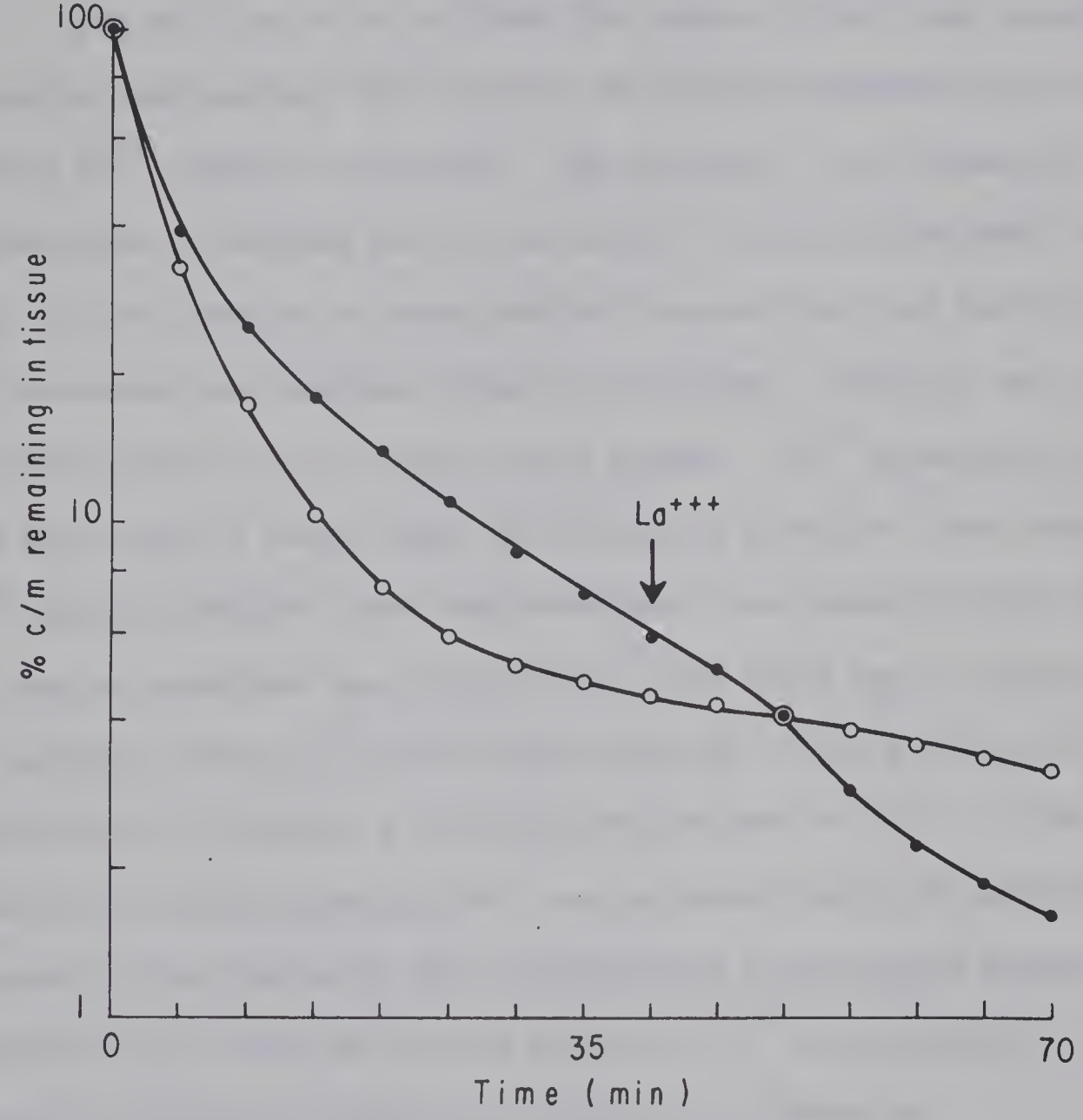


Figure 7. Effect of La on the loss of Ca^{45} from aortic rings.

Rings were incubated in NKR (Ca^{45}) then washed out into Ca-free (EGTA) NKR with La present throughout the efflux (o), or La added after 40 minutes of washing (•).

D. Blockade of Ca^{45} Uptake by La

The ability of La to block the uptake of Ca^{45} was tested by comparing the residual Ca^{45} content of tissues incubated in La and La-free Ca^{45} labelled solutions. The procedure for incubation was as described in Methods but La was added to the labelled NKR 3 minutes prior to the transfer of experimental tissues from this solution to the La-containing labelled stimulant solutions. Controls were not incubated with La at any time during uptake. Ca^{45} determinations were made after a 1 hour wash in Ca-free,La solution. The residual Ca^{45} contents derived from this experiment are shown in Table II. La clearly inhibited the uptake of Ca^{45} and there was no difference in residual tissue Ca^{45} when uptake occurred in the presence of La irrespective of whether a stimulant was present or not. In the absence of La the uptake of Ca^{45} was increased under HK stimulation compared to the uptake of NA stimulated and unstimulated tissues. Further study on the HK induced uptake of Ca^{45} was conducted, the results of which are reported in Section E of Results.

TABLE II

Effect of La on the uptake of Ca^{45} (mmoles/kg) †

Uptake Medium	No La (control)††	n	La*	n
NKR	0.0924 ± 0.0114	4	0.0256 ± 0.0019**	4
NKR + NA 4 x 10^{-6} g/ml	0.1003 ± 0.0089	4	0.0262 ± 0.0015**	4
HKR	0.2566 ± 0.0501	4	0.0249 ± 0.0025**	4

† one of two experiments.

†† All times washed in Ca-free(EGTA)NKR for 40 minutes followed by 20 minutes in the same solution containing 2mM La (see Methods, section 2a).

* All times washed 1 hr in La (2mM) containing Ca-free(EGTA)NKR after incubation.

** Significant difference ($p<.05$) between control and La treated tissues.

E. Tension Studies: The Effect of La^{+++} on HK and NA Contractures

In light of recent reports (van Breemen and DeWeer, 1970), and evidence presented in the present study suggesting that La^{+++} blocks the influx of Ca across the cell membrane La^{+++} was used as a tool to resolve the source from which Ca is utilized during the phasic and tonic stages of the HK and NA contractures of rabbit aorta rings. Previous reports implicate both extracellular and intracellular Ca pools as sources of contractile Ca (Hinke, 1964; van Breemen, 1969). Therefore using La^{+++} to block transmembranal Ca fluxes it should be possible to differentiate the relative contribution of these pools to the HK and NA contractures provided La does not affect internal releasible Ca. van Breemen (1969) reported that La^{+++} (1.8mM) was capable of relaxing the NA contracture but not the HK one. Furthermore La^{+++} added prior to stimulation abolished the phasic stage but not the tonic stage of the HK contracture whereas it abolished the tonic but not the phasic stage of the NA contracture. Attempts to reproduce these results were not entirely successful. The La^{+++} concentration in all the present studies was 2mM. At this concentration La^{+++} could not only relax the NA contracture (NA 10^{-6}g/ml) but also the HK one (Figure 8, a and b). This observation was consistent in all tissues studied (n=15) and indications were that an approximate 10-fold reduction in La^{+++} concentration to 0.1 or 0.3mM (n=4) could also relax or at least cause a partial relaxation of the HK contracture (Figure 9). Since the bath solutions used in this study were not identical to those used by van Breemen, the possibility arose that the ability of La^{+++}

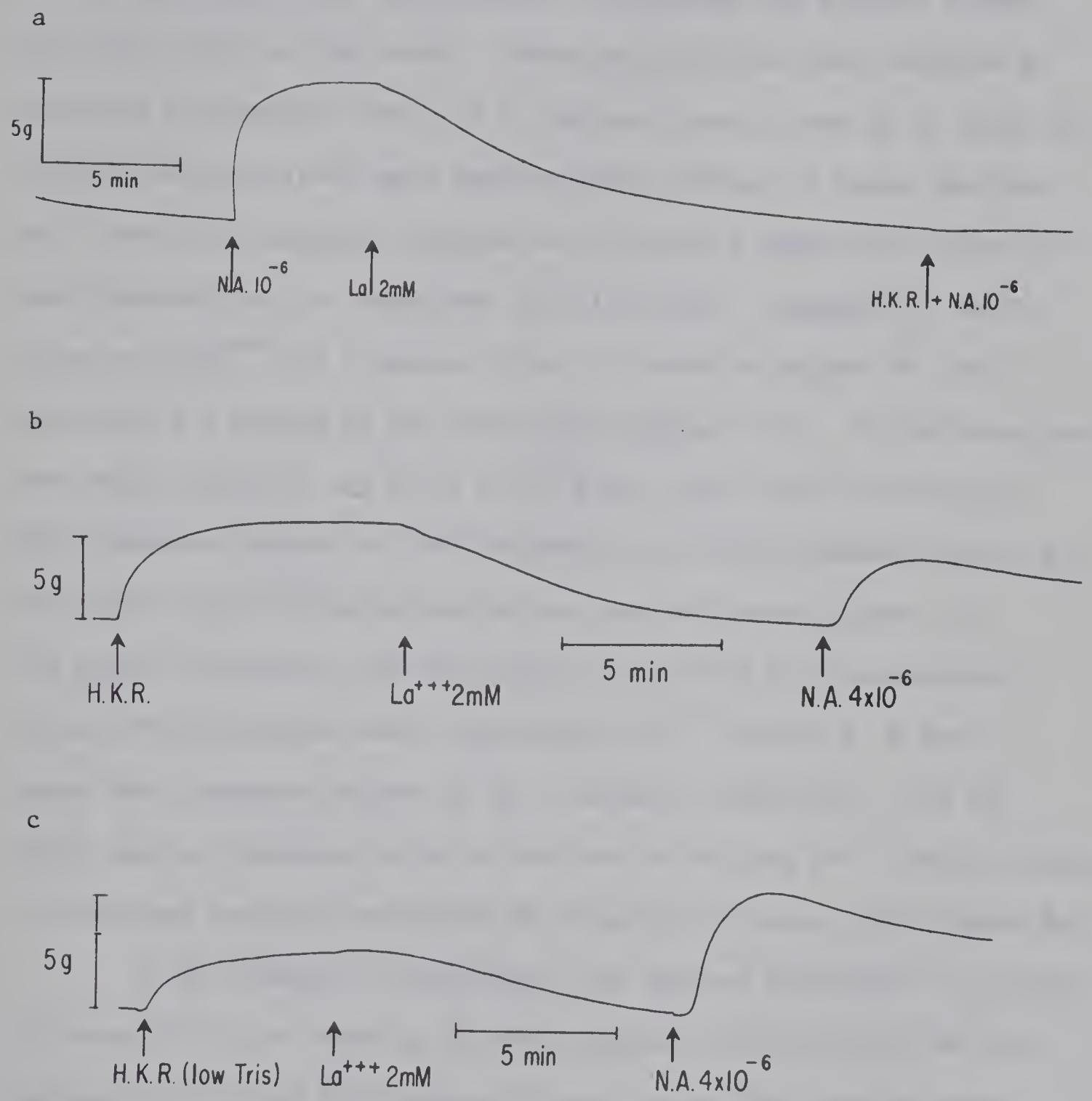


Figure 8. Effect of La on NA (10^{-6}g/ml) and HK contractures of rabbit aorta.

In the lower trace (c) the tris buffer concentration was lowered from 21.9 to 5mM and glucose lowered from 50 to 10mM (isomolarity was maintained with NaCl) and responses compared to trace (b). Tissues were from the same animal in this comparison.

(HK=HKR)

to relax the HK contracture was a function of the concentration of solution constituents, particularly tris-buffer and glucose (These were both high in this study). When the solutions were modified by reducing tris-buffer from 21.9 to 5mM and glucose from 50 to 10mM, the results obtained (n=4) were qualitatively similar to those obtained with unmodified solution (Figure 8c); therefore unmodified solutions were retained for the remainder of this study. Treatment of aortic rings with La^{+++} for 3 minutes prior to stimulation with HK (n=4) abolished all stages of the contracture (Figure 10). On the other hand the phasic stage of the NA (4×10^{-6} g/ml) could still be elicited. This response decayed in 10-15 minutes to pre-drug tension levels, i.e. the tonic stage of the NA contracture was abolished (Figure 10). The phasic response to NA could also be obtained in HK stimulated rings (n=6) which had been relaxed with La^{+++} (Figure 8, b and c). Again this response decayed in 10-15 minutes (Figure 10). On the other hand no response could be obtained by raising the K concentration of solutions bathing La-relaxed NA stimulated tissues (n=3)(Figure 8a).

In an attempt to demonstrate the implied similarity of action of adding La to or removing Ca from tissues in contracture, Ca was replaced with 0.5mM EGTA during HK (n=4) or NA (n=4) contractures. Tension fell to baseline levels within 20-30 minutes as observed in La^{+++} treated tissues; furthermore, additions of NA (n=4) to relaxed rings in HKR could elicit a phasic response of 10-15 minutes duration (Figure 11, a and b). NA (n=6) could also induce a further increase in tension of aortic rings maximally contracted to K ion (n=6)(Figure 12). Such observations have been reported in the past for several

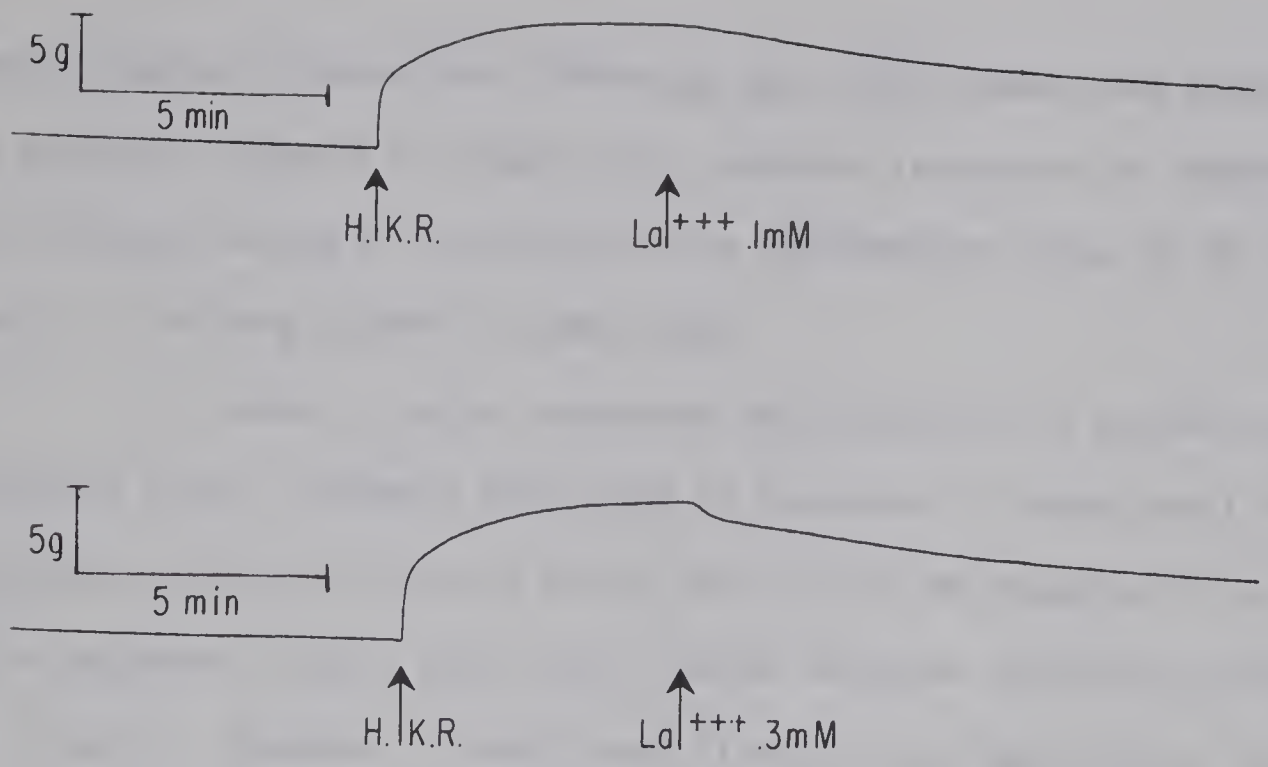


Figure 9. Effect of 0.1 and 0.3mM La on the HK contracture. (HK=HKR).

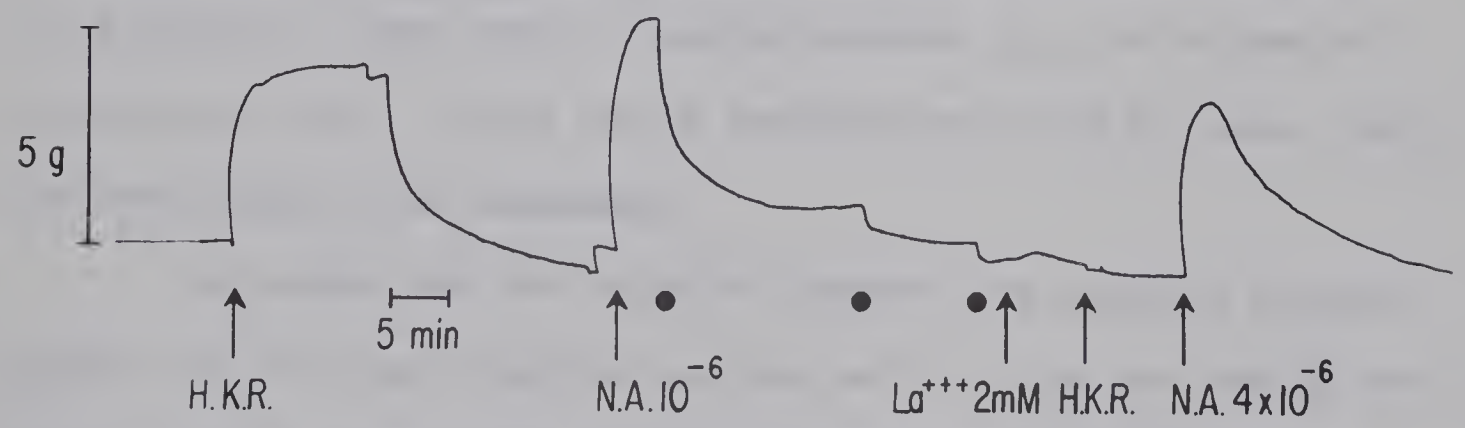


Figure 10. Effect of La exposure prior to stimulation on HK or NA contractures of rabbit aorta.

Tissues were exposed to La for 3 minutes prior to stimulation with HK or 4 x 10^{-6}g/ml NA (HK=HKR).

(●) Bath fluid replaced with NKR.

smooth muscle preparations (Evans et al., 1958; Edman and Schild, 1961). In addition, Figure 12 shows that a similar increment in tension could be achieved during a contracture to a supramaximal dose of NA (10^{-6} g/ml) by exchanging NKR for HKR (n=4).

In tension studies conducted approximately 12 months after those reported above, attempts were made to determine if additional NA responses could be elicited after the initial NA response in La relaxed HK stimulated tissue, i.e. after the NA response depicted in Figure 8, b and c. However, it was found firstly, that many (25 of 30) HK contractures did not relax fully (50-80%) on exposure to La 2mM as they had done in the previous studies, and secondly, that NA responses elicited after these relaxations would not relax as they had done 12 months previous. The effect of La on NA contractures appeared to be similar in both sets of tension studies, i.e. La relaxed NA contractures fully. Since the HK contractures would not relax fully, the investigation was abandoned.

The reason for the variation between this group of tension studies and the ones conducted earlier was not clear but taking into account errors in solution preparation and tissue handling the variation appears to be biological and, if so, may account for the lack of any La effect on HK contractures in van Breemen's study (1969).

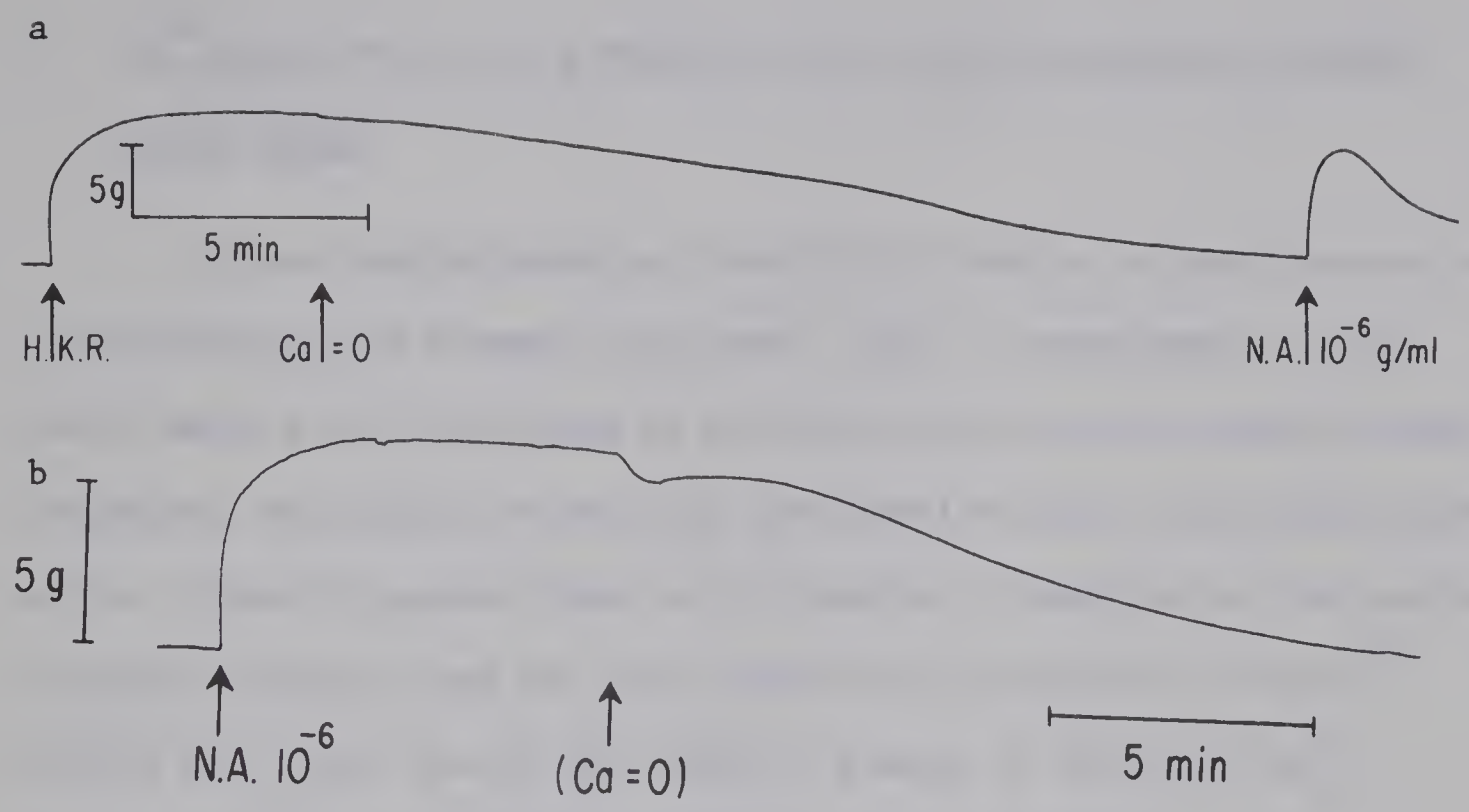


Figure 11. Effect of Ca depletion during NA and HK contractures of rabbit aorta.

Ca was removed from stimulant media during (a) HK (HKR) contracture or (b) during NA (10^{-6}g/ml) contracture. Ca was replaced by 0.5mM EGTA.

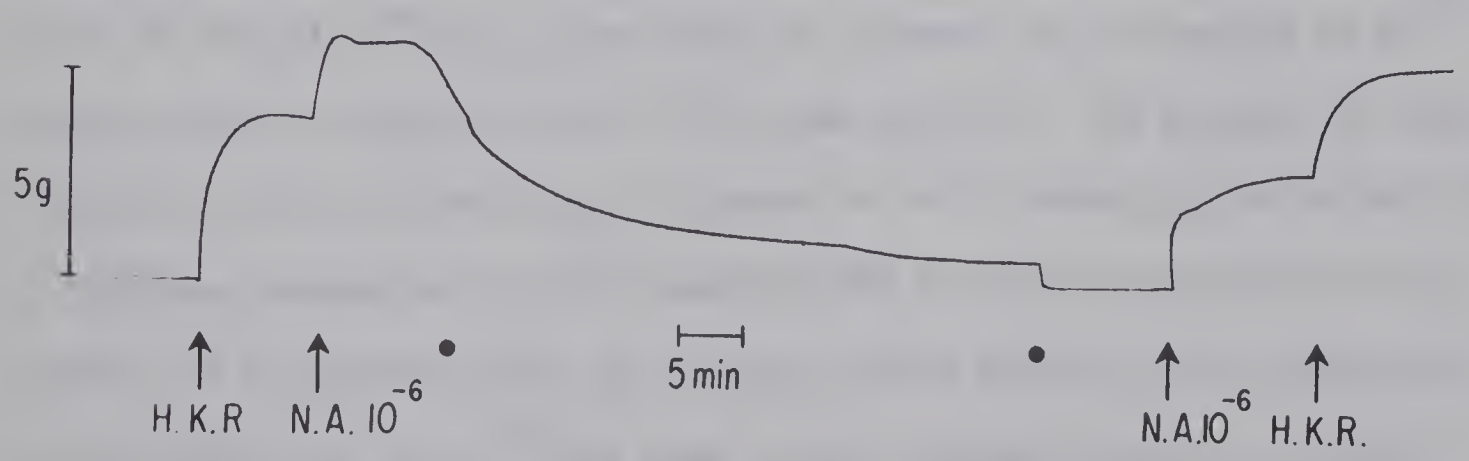


Figure 12. The effect of simultaneous stimulation of rabbit aorta with NA (10^{-6}g/ml) and HK (HKR).

(•) Bath fluid replaced with NKR.

F. Ca^{45} Mobilization as a Result of HK or NA Stimulation of Rabbit Aortic Rings

It has been pointed out that 90% of the Ca in rabbit aorta is extracellular (van Breemen and Lesser, 1971). Measurements of the small amounts of Ca utilized in excitation contraction coupling would, therefore, be subject to error by contributions from this large source of Ca. Since it appears that La is capable of removing Ca from extracellular binding sites and also capable of blocking the late Ca^{45} efflux from cells it was used here as a means of resolving Ca^{45} movements during contractures stimulated by HK and NA.

The figures in Table IIIa (see also Table II, controls) represent the residual Ca^{45} concentration of tissues loaded as described in Methods for 1 hour in stimulant and control solutions labelled with Ca^{45} then washed for 1 and 3 hours in Ca-free(EGTA)NKR (all Ca-free solutions contained EGTA 0.5mM in these experiments) made 2mM in La. Both HK and NA 10^{-6}g/ml stimulated an increase in the uptake of Ca^{45} which could be detected after both wash periods. The figures in Table IIIb show that a significant increase in Ca^{45} content occurs after only 5 minutes incubation in Ca^{45} labelled HKR but the net uptake is only about 1/3 (0.040 mMole/kg) of the net uptake after 1 hour incubation (0.137 mMole/kg) in Ca^{45}-HKR even though maximum tension is reached after 2-3 minutes of HK stimulation. The HK induced increase in Ca^{45} uptake was much greater than that induced by NA. Similar results were obtained by van Breemen and Lesser (1971).

Ca^{45} was also elevated in HK stimulated tissues washed for 20 or

TABLE III

Effect of NA and HK on the uptake of Ca^{45} (mmoles/kg)

Uptake Medium	Ca^{45} content after La wash for 1 hr	n	3 hr	n
a				
(Control) NKR	0.1135 ± 0.0037	16	0.0496 ± 0.0015	8
NKR + NA 10^{-6} g/ml	0.1483 ± 0.0092*	16	0.0587 ± 0.0024*	8
(Control) NKR †	0.0906 ± 0.0013	4	0.0362 ± 0.0014	4
HKR †	0.2281 ± 0.0111*	4	0.1452 ± 0.0130*	4
b				
(Control) NKR	0.1274 ± 0.0044	6	---	
HKR	0.1675 ± 0.0049*	6	---	
	20 min.(Ca-free NKR+EGTA)		60 min (Ca-free NKR+EGTA)	
c				
(Control) NKR	0.3539 ± 0.0469	8	0.1365 ± 0.0116	8
HKR	0.6365 ± 0.0831*	8	0.2283 ± 0.0240*	8

[a] Ca^{45} uptake as described in Methods. Experimental tissues stimulated for 1 hr.

[b] Ca^{45} uptake as described in Methods. Experimental tissues stimulated for 5 min.

[c] Ca^{45} uptake as in a but washed in La-free NKR containing 0.5mM EGTA for 20 and 60 minutes.

* Difference between control and stimulated tissue Ca^{45} content significant ($p<.05$)

† one of two experiments.

60 minutes in EGTA Ca-free Krebs in the complete absence of La (Table IIIB). A similar observation has been reported by Sperelakis (1962) in uterine smooth muscle using an unlabelled Ca-containing Krebs wash for 60 minutes.

The uptake of Ca^{45} in HK stimulated aorta was further increased by addition of NA 10^{-6}g/ml to the Ca^{45} labelled HKR (Table IV). These tissues were washed for 1 hour in a La containing, Ca-free NKR solution after incubating in stimulant solutions for the usual 1 hour period and also a 2 hour period.

The status of the HK-induced increase in Ca^{45} is subject to question on the grounds that in tension studies (Results, section D) both Ca removal from or La addition to the HK contracted aortic rings relaxed the contracture in 20-30 minutes yet in the above Ca^{45} uptake experiments the Ca^{45} content remained elevated after 60 and 180 minutes of La wash. Furthermore, the decrease in the net gain of Ca^{45} occuring between 60 and 180 minutes is smaller in HK stimulated tissues than in NA or unstimulated tissues. These observations suggest that the increment in Ca^{45} is not free to interact with the contractile proteins at termination of washout as implied by van Breemen (1969). To further clarify the nature of the HK induced increment in Ca^{45}, experiments were conducted to ascertain whether any binding sites were sensitive to K or NA thereby giving some indication of the availability of Ca to contractions induced by these agents.

Tissues labelled in Ca^{45} HKR were washed in Ca-free HKR with La or in Ca-free NKR with La for 1 and 3 hours. The residual Ca^{45} contents were then compared for each wash solution. Similarly,

TABLE IV

Effect of simultaneous stimulation with HK and NA on Ca^{45} uptake (mmoles/kg)†

Uptake Medium	Duration of uptake 1 hr	n	2 hrs	n
(Control) HKR	0.2236 ± 0.0113	8	0.2563 ± 0.0169	8
HKR + NA 10^{-6} g/ml	0.3206 ± 0.0173*	8	0.3614 ± 0.0183*	8

†All tissues washed in 2mM La, Ca-free(EGTA)NKR for 1 hr.

*$p<.05$ compared to HKR alone.

tissues labelled in HKR were washed in Ca-free La-containing NKR with and without NA 4 x 10^{-6} g/ml and their Ca^{45} contents compared to the controls (Table V). This increase could only be observed after 3 hours of washing. The results of the HK wash suggest a blockade of Ca^{45} efflux which might be applicable as a means of raising intracellular free Ca during HK stimulation. To check this possibility, the HK wash experiment was repeated using Ca-containing La-free solutions (La was added during the last 20 minutes of each time period to remove extracellular Ca^{45}) thereby reducing the effects of Ca-lack and La on Ca^{45} efflux. Qualitatively similar results were obtained with these solutions as with Ca-free, La-containing solutions (Table Vb). When the La-containing Ca-free HKR was again used to ascertain its effects on tissues loaded in Ca^{45} labelled NKR, the differences in Ca^{45} content between experimental and control tissues was insignificant (Table VI).

The observations that the NA response in La-relaxed HK depolarized aorta was only phasic (Fig. 10), despite the presence of N_A, and that a NA containing wash does not alter the rate of efflux of the HK induced increase in Ca^{45} (even though tension is developed under these circumstances), suggests that either Ca^{45} is being rebound within the cell or that Ca^{45} is lost to the bathing medium upon relaxation.

To test these possibilities, tissues were incubated for 2 hrs in Ca^{45} labelled NKR, but during the first 10 minutes of this period the tissues were exposed to NA 10^{-6} g/ml. This was done to make sure that NA releasable Ca was fully exchanged with Ca^{45}. Following the incubation period, the tissues were washed in La-containing NKR or the same

TABLE V

Effect of HK and NA containing washes on the HK induced increment in Ca^{45} (mmoles/kg)*

Wash Medium	Ca^{45} content after wash for 1 hr	n	3 hr	n
a†				
(Control) Ca-free NKR + La	0.3375 ± 0.0166	4	0.0964 ± 0.0025	4
Ca-free HKR + La	0.3595 ± 0.0345	4	0.2307 ± 0.0134††	4
(Control) Ca-free NKR + La	0.2343 ± 0.0139	4	0.0705 ± 0.0046	4
Ca-free NKR + NA 10^{-6}g/ml + La	0.2323 ± 0.0250	4	0.0730 ± 0.0061	4
b†				
(Control) NKR	0.1774 ± 0.0045	4	0.0373 ± 0.0013	4
HKR	0.1856 ± 0.0117	4	0.0686 ± 0.0028††	4

* Uptake as described in Methods. All tissues incubated in HKR (Ca^{45}) for 1 hr.

a La (2mM) in wash media for duration of each wash period.

b La (2mM) added during the final 20 minutes of each time period to remove remaining extracellular Ca^{45}.

† one of two experiments.

†† $p<.05$ compared to control.

TABLE VI

Effect of HK washes on the Ca^{45} content of rabbit aorta (mmoles/kg)*

Wash Medium	Duration of La Wash 1 hr	n	3 hrs	n
(Control) Ca-free NKR + La	0.0992 ± 0.0005	4	0.0553 ± 0.0006	4
Ca-free HKR + La	0.1065 ± 0.0075	4	0.0526 ± 0.0013	4

* one of two experiments. All tissues were incubated in NKR Ca^{45} for 2 hrs.

solution containing NA 4 x 10^{-6} g/ml. This latter solution is identical to that causing the phasic response to NA (Figure 10) and to that causing relaxation of the NA contracture (Figure 8).

The difference in Ca^{45} content between control tissue and tissues washed in NA-containing solutions was not significant after 20 minutes, but was after 60 minutes ($p < .05$) of washing. However, the small amount of Ca^{45} that would theoretically be lost during relaxation raises the question as to whether these results are meaningful. This point will again be raised in the Discussion.

TABLE VII

Effect of a NA containing wash on the Ca^{45} content of rabbit aorta (mmoles/kg)

Wash medium	Ca^{45} content after wash for 20 min.	n	60 min.	n
(Control) NKR + La	0.1541 ± 0.0076	12	0.1349 ± 0.0112	12
NKR + NA 4 x 10^{-6} g/ml + La †	0.1455 ± 0.0097	11	0.1071 ± 0.0063*	12

† These tissues were exposed to NKR (Ca^{45}) + NA 10^{-6} g/ml for 10 minutes then incubated in NKR (Ca^{45}) for the remainder of a 2 hr uptake period.

* $p<.05$

IV. DISCUSSION

The aims of the present investigation were two-fold: the first aim was to test the assumptions concerning the actions of La that make this ion a potentially useful tool in measuring the intracellular Ca compartments during contractions of smooth muscle. These assumptions are that,

1) La releases extracellular bound Ca,

2) La blocks the influx of Ca into the cell,

3) La blocks the efflux of intracellular Ca,

and, 4) La has no other actions which could account for its effects on contractility.

The second aim, assuming the first aim to be fulfilled, was to use the actions of La as a tool in the resolution of Ca movements during HK and NA induced contractions of rabbit aorta.

A. The Actions of La

Electron micrographs of vascular smooth muscle have shown that colloidal La does not penetrate the cell (Devine and Somlyo, 1970) but evidence has been presented suggesting that La in the ionic rather than colloidal form does enter the cell of uterine smooth muscle (Hodgson, 1971). Nevertheless, the reduction of tissue Ca after washing in ionic La solutions has been considered due to displacement of extracellular bound Ca (Weiss and Goodman, 1969; Sanborn and Langer, 1970; van Breemen and McNaughton, 1970; Goodman and Weiss, 1970). The present study, in part supports the above authors views judging from the La induced increase in the rate of Ca^{45} efflux into both Ca-free and Ca-free EDTA containing NKR. In the presence of the specific Ca chelator EDTA, La displaces Ca^{45} in addition to that displaced by EGTA. Since the displacement of this Ca^{45} is also observed in the presence of NA, the Ca^{45} displaced does not appear to be derived from the NA releasable Ca pool. Ca released by NA has been postulated to be intracellular (Hinke, 1965; van Breemen et al., 1969, 1971). This suggests that La is displacing Ca from an extracellular site not accessible to EGTA as well as sites accessible to EGTA. However, the possibility that La might displace intracellular Ca unrelated to the NA releasable Ca pool has not been ruled out.

The 10-15 minute delay in the onset of La action in the presence of EGTA might be explained on the basis of time required for intracellular penetration of La and subsequent loss of Ca^{45} or on the basis of time for La penetration into an extracellular region which

is not accessible to EGTA because of its size. According to these explanations, after prolonged exposure to EGTA La would have no effect. This prediction was not tested in the present study.

Blockade of membrane Ca transport by La has been shown directly in barnacle muscle fibres (Hagiwara and Takahashi, 1967), lobster axon (Takata, Pickard, Lettvin and More, 1966), artificial membranes (van Breemen and van Breemen, 1969) and in squid axon (van Breemen and DeWeer, 1970) but only indirectly in rabbit aorta (van Breemen, 1969). The results of the present study clearly demonstrate that La blocks the uptake of nearly all Ca^{45}. The small uptake (0.02 mMoles Ca^{45}/kg) in the presence of La may indicate that the La block is incomplete. Nevertheless, no tension changes occur during HK stimulation of La exposed tissues. Since such changes are presumably mediated by an influx of extracellar Ca (Hinke, 1965; van Breemen, 1969) it appears that any La independent influx of Ca^{45} is not sufficient to activate contractile proteins or is directed to a compartment where it cannot activate contractile proteins. If the control Ca^{45} values from Table II are taken to be 100%, the blockade produced by La can be calculated to be 80% in both NA stimulated and unstimulated tissues and 90% in HK stimulated tissues. These values are based on the assumption that Ca^{45} contents represent intracellular Ca.

In addition to the blockade of Ca^{45} influx the results of the present investigation suggest that La diminished efflux of intracellular Ca^{45}. Tissues washed sufficiently long with La exhibited elevated residual Ca^{45} contents compared to control tissues. However, if the difference in Ca^{45} constitutes the La blocked intracellular Ca^{45} then

it can be calculated that intracellular Ca^{45} decreases by roughly 50% when La washing is extended from 1 to 3 hours. It appears, therefore, that La may not completely block the loss of intracellular Ca. A similar conclusion has been reached by van Breemen in a recent report (van Breemen *et al.*, 1972). He calculated the blockade if Ca^{45} efflux to be 50%.

B. Tension Studies

The hypotheses concerning the derivation of Ca sustaining K and drug contracture have been discussed in the Introduction (sections D and E) but can be summarized into two general and competing explanations of excitation-contraction coupling in smooth muscle. The first explanation is that K stimulates an increased influx of extracellular Ca while stimulant drugs release intracellular bound Ca (Daniel, 1963; Hinke, 1964, 1965; Daniel and van Breemen, 1966; van Breemen, 1969; van Breemen et al., 1971). The second explanation is that both K and stimulant drugs induce an increased influx of extracellular Ca but that drugs are more effective in this action (Somlyo and Somlyo, 1968). Within the frame-work of these explanations and the actions of La already discussed, proposals will be made in the subsequent discussion to explain the results of tension studies and Ca^{45} movements during stimulation of rabbit aortae.

The contractures induced in this study to both HK and NA are two stage: an initial phasic stage in which tension quickly develops, followed by a tonic stage in which there is little or no change in tension. Exposure to La or removal of Ca during the tonic stage relaxes both HK and NA contractures suggesting that the tonic stage of both responses is maintained by an influx of extracellular Ca. However, van Breemen (1969) reported that La relaxed the NA but not the HK contractures of rabbit aorta. He, therefore, concluded that the tonic stage of the HK response was sustained by release of intracellular bound Ca.

The reason for the different actions of La in van Breemen's and the present study is not clear. The initial set of tension experiments, from which the records have been taken for this presentation were conducted at various times during the year without changes in the effect of La on HK contractures. This suggests that seasonal changes in rabbit vasculature are not responsible. Nevertheless, tension studies conducted 12 months after the termination of the initial set did show variation in La effect on the HK contracture; i.e., contractures would not relax fully on exposure to La. This latter effect is assumed to be related to biological variation in the rabbits partially unrelated to seasonal changes.

Variations in solution constituents were also tested but found not to be a causitive factor in the variation of La effect on HK contractures.

The observation that La exposure prior to stimulation abolishes the HK contracture and the tonic, but not the phasic, stage of the NA contracture suggests that all stages of the HK response depend upon influx of extracellular Ca while the phasic stage of the NA response depends upon release of intracellular Ca. Also in favor of intracellular release of Ca by NA are the observations that the NA response can be obtained in tissues in which the HK contracture has been relaxed either by exposure to La or by removal of Ca and that NA can increase tension in tissues responding maximally to HK. Not only do these observations suggest intracellular release of Ca by NA but also that this Ca is not acted upon by HK. Hudgins and Weiss (1968) and Hinke (1965) have also noted in vascular smooth muscle that NA appears to affect firmly bound Ca stores that are little affected by K ions.

C. Ca^{45} Movements during HK and NA Stimulation

The use of the La wash to remove extracellular Ca and partially block the efflux of intracellular Ca reveals an increased uptake of 0.137 mMoles Ca^{45}/kg wet weight in tissue exposed to HK for 1 hour and washed in La for 1 hour. However, in tissues exposed to NA for 1 hour and washed in La for 1 hour the increased uptake is only 0.0345 mMoles Ca^{45}/kg wet weight, even though the tension developed to both these agents is similar. Similar results have been obtained by van Breemen and Lesser (1971) also in the rabbit. These authors consider their results as evidence to support the theory that HK but not NA stimulates an influx of extracellular Ca^{45}. The increased Ca^{45} contents found in this study when considered alone, support this theory but when they are put into context with certain other observations both from this and other studies, it appears that the above theory is oversimplified. These observations are mentioned in the following paragraphs.

In this study,tension experiments done in conjunction with uptake experiments,reveal that tissues exposed to La either in the presence or absence of Ca are relaxed at times when Ca^{45} is found to be elevated. This suggests that Ca^{45} is not in the free or active form but bound within the cell. Furthermore, it appears that La blockade of Ca^{45} efflux may not be responsible for maintaining elevated Ca^{45} in HK stimulated tissues since it is elevated in those HK treated tissues washed only in Ca free(EGTA)NKR. Similarly, Sperelakis (1962) has observed increased Ca^{45} content in cat intestine after the tissue was exposed to K and washed for 1 hour in Ca containing

NKR, a solution which would quickly remove free or loosely bound Ca^{45}. The observations cited from the present study suggest that the Ca^{45} uptake as a result of HK stimulation is not all related to tension development particularly not after La washing. In connection with this latter point is the finding both in this and van Breemen's studies (1970, 1971) that the uptake of Ca^{45} continues even after maximum tension has been reached (see Table IIIa and IIIc) under HK stimulation. This implies two things: 1) either the Ca^{45} taken up after maximal tension is reached is bound to some intracellular sink and therefore, is inactive, or that 2) the exchange between Ca^{40} and Ca^{45} is not in equilibrium during the exposure to labelled HKR. However, total Ca and Ca^{45} determinations in this study indicate that exchange is 100%. Furthermore, Hodgson, using the La wash, could detect no increase in Ca^{45} content on exposure of uterine tissue to isotonic K (140mM) solutions, although increased uptake was observed in hypertonic solutions. These results indicate some other factor rather than Ca^{40}/Ca^{45} exchange is important in the uptake of Ca^{45} during HK stimulation.

One further consideration which favors the idea of a Ca sink is the fact that the net Ca^{45} uptake appears to be larger than that required for maximal activation of contractile proteins.

Fuchs and Briggs (1968) have estimated that 1.3μMoles Ca/g of myofibrillar protein would be bound to skeletal muscle myofibrils during contraction in the presence of 10^{-6}M Ca. This Ca has been found to bind mainly to troponin at physiological Ca concentrations, *i.e.* 10^{-6}M. Troponin constitutes roughly 10% of myofibrillar proteins.

In glycerinated smooth muscle (vascular) 10^{-6}M Ca is required to produce maximal tension (Filo, Ruegg and Bohr, 1965) and in cow aorta, purified but Ca-sensitive actomyosin (i.e. containing tropomyosin and troponin) has been prepared in a yield of 2.5 mg/g wet tissue (Sparrow, Maxwell, Ruegg and Bohr, 1970). If it is assumed that troponin constitutes 10% of vascular smooth muscle actomyosin and is similar in Ca binding properties to skeletal muscle troponin, then 2.5 mg actomyosin would bind $2.5 \times 10^{-6} \times 1.3 = 3.3\mu$Moles Ca/kg wet tissue. However, Ca^{45} content after 1 hour La wash is 0.137mmoles/kg. This value appears to be roughly 40 times larger than that required for maximal activation of contractile proteins. Furthermore, even if part of this Ca^{45} were directly involved in activation of contraction it is not sufficient to cause maximal activation of protein since extra tension can be generated in HK contracted tissues by addition of NA. In light of the above observations and considerations it seems reasonable to suggest that during the HK induced influx of Ca^{45} part is bound to troponin while concurrently part is bound to some intracellular sink. This Ca sink does not appear to bind Ca during NA stimulation judging from the relatively small uptake of Ca^{45} during NA stimulation as compared to that during HK stimulation but the reason for this may be more complex than the simple postulate that NA does not alter the Ca permiability of the cell membrane (van Breemen et al., 1971).

There is good evidence to suggest that part or all of the calcium sustaining the NA contracture is derived from extracellular sources. Both in this, and in van Breemen's study (1969), La relaxed the tonic

stage of the NA contracture and prevented the development of the tonic stage of the NA contracture when the tissues were exposed to La prior to stimulation. Waugh (1964) has shown that responses to bolus injections of $CaCl_2$ are enhanced after a brief exposure to either NA or K (60mM). Waugh's observations suggest that both K and NA have a similar effect in increasing the amount of extracellular Ca reaching the contractile proteins presumably by increasing the permiability of the cell membrane to Ca. Hiraoka, Yamagashi and Sano (1968) also noted that NA contractions of rabbit ear artery in normal Ca but Na free solutions did not relax after removal of NA but did so if external Ca was removed. These authors suggest this as clear evidence for involvement of extracellular Ca in this response. If NA, like HK does indeed increase the permiability of the cell membrane then an alternative explanation to that offered by van Breemen (1971) must be sought to explain the lack of Ca^{45} uptake in tissues exposed to this drug. An alternative is suggested in the following paragraph.

The Ca sink proposed earlier in this discussion to explain the increased uptake of Ca^{45} as a result of HK stimulation may well function only in the presence of elevated concentrations of the K ion, thus, theoretically although NA may increase influx of extracellular Ca into the cell, in the absence of elevated K this Ca is not taken up by the sink but is possibly lost during the La wash procedure by effluxing past the incomplete La block, resulting in low residual Ca^{45} content. The idea that K but not NA may effect an intracellular Ca sink is further supported by the findings that the net Ca^{45} uptake induced by NA is larger in the presence (0.097mMole/kg) than in the absence (0.0348mMole/kg)

of HK even though, according to the results of Somlyo (1968), (but not clearly demonstrated in the present study) tension development to NA is very similar whether stimulation occurs in polarized or depolarized (K=179mM) tissues; and by the fact that HK but not NA containing washes decrease the rate of loss of Ca^{45} during efflux from the HK induced Ca^{45} pool.

An alternative explanation for the effect of HK wash is that K blocks the efflux of intracellular Ca thereby increasing intracellular Ca. This Ca might then support the HK contracture. However, unstimulated Ca^{45} labelled tissues exhibit no increase in Ca^{45} content when washed in HK solutions. Furthermore, since HK contractions are abolished by prior exposure of the tissues to La, it appears that increased influx of extracellular Ca is more feasible than the slowing of intracellular Ca efflux during these contractures. The latter mechanism also implies that intracellular Ca would participate in activation of contractile proteins and would, therefore, presumably be independent of La action.

The size of any NA induced Ca influx compared to that induced by HK is difficult to determine. The increase in the uptake of Ca^{45}, and also tension, produced by NA stimulation of HK contracted tissues suggests that NA increases Ca^{45} influx over and above that caused by HK. However, the increase in tension might be explained in terms of release of intracellular Ca but this would not explain the increase in Ca^{45} uptake induced by NA in HK media for if intracellular Ca were solely involved there would be no net increase in Ca^{45} uptake during NA stimulation of HK stimulated tissues. It seems likely, therefore,

that both intracellular release of Ca and extracellular efflux of Ca^{45} are involved.

Similar to the increase in tension produced by NA in HK treated tissues, HK increases tension in tissues contracted to supramaximal doses of NA. This suggests that HK increases Ca influx to a greater extent than NA and is, therefore, in conflict with the ideas concerning the NA stimulated Ca influx. However, the conflict may be overcome by suggesting that Ca influx is mediated by two different channels; one utilized by drugs, such as NA and one utilized by HK. This idea seems reasonable in light of Hinke's (1964) observation that in Ca depleted rat tail artery the relationship between tension development and external Ca concentration is linear for HK but complex for NA stimulated tissues and in light of Somlyo's results (1968) that responses obtained by supramaximal doses of angiotensin, vasopressin, and adrenaline are remarkably similar in magnitude whether elicited in polarized or depolarized (179mM K) tissues. Nevertheless Somlyo's results could also be explained in terms of intracellular release of Ca by drugs.

Although the present investigation was not specifically designed to study the disposal of Ca during relaxation of rabbit aorta, three observations imply the presence of a Ca extension pump in this tissue that may take part in relaxation. The first observation is that tension produced with a supramaximal dose of NA or with HK is not the maximal tension the tissue can develop. Although this may appear to be unrelated to relaxation processes, it does imply that the supply of activating Ca is halted before maximal activation of contractile proteins is reached even though Ca influx is not halted, particularly with HK

stimulation. The second observation is that the response to NA of tissues exposed to La is only phasic despite the continued presence of NA. Similarly the NA contracture relaxes on exposure to La despite the presence of the drug. It is assumed in both cases that Ca released by NA is not rebound (thus causing relaxation) to the site, from which it was released, while NA is present. The third observation is that La blockade of intracellular Ca^{45} efflux is only partial. In a very recent study (van Breemen et al., 1972) this blockade has been estimated to be only 50%.

Results of studies conducted to determine the loss of Ca^{45} (if any) during the relaxation of the phasic NA response occurring in La exposed tissues were difficult to interpret. The NA phasic response decays in 10-15 minutes yet Ca determination after 20 minutes revealed no significant difference in Ca content. Presumably at this time Ca should have been extruded according to tension records. Nevertheless, Ca determination after 1 hour revealed a significantly decreased Ca content (0.0247mMoles/kg) as might be expected if extrusion occurs.

Consideration of the small amount of Ca^{45} in terms of cpm moved during relaxation further accentuates the problems of interpreting such results. Assuming, during relaxation, 3.3μMoles Ca/kg tissue is removed from contractile proteins (this value was derived earlier in the discussion), the cpm associated with this concentration of Ca would be 3 cpm/mg (the specific activity of the media used in the above labelling solution was 5×10^5 cpm/μMole Ca) but the residual tissue Ca^{45} after 20 minutes of La wash has a mean value of 65 cpm/mg (range 50-80 cpm/mg).

D. Conclusions

From consideration of the data presented in this study, the following conclusions are drawn.

1) La displaces extracellular Ca while blocking the influx and efflux of intracellular Ca. The blockade of Ca efflux is not complete and further studies are required to determine the extent of changes in this parameter. No real evidence has been presented in this and other studies to draw conclusions as to whether or not the La ion enters the cell. If La is to be of any use as a tool in the determination of cell Ca fluxes this latter point must be clarified.

2) The phasic stage of the NA contracture is maintained by release of intracellular bound Ca while the tonic stage utilizes Ca influxing from extracellular sources.

3) Both the phasic and tonic stage of the HK contracture are maintained by an influx of extracellular Ca. Furthermore the NA releasible bound Ca is not acted upon by HK.

4) Only a portion of the HK induced increase in Ca influx is utilized to develop tension; the remainder is bound at an intracellular sink.

5) Potassium ion but not NA may be capable of increasing Ca binding during stimulation despite its net action of increasing the concentration of intracellular free Ca.

Although La appears at this point in time to be a useful tool in the study of intracellular Ca fluxes, there are two main questions concerning its actions; they are:

1) does La enter the cell?

2) does La injure the tissue?

It has already been pointed out that electronmicrographs are of no use in determining the answer to question 1 because, in order to 'see' La, it must be in a colloidal form and not the desired ionic form. It would appear more reasonable to follow the method of Hodgson (1971) in which La 140 distribution in subcellular fractions was studied. He concluded that La did enter uterine smooth muscle cells.

The effects of La on tension development in aorta is irreversible thus control responses to test the viability of the tissue at the termination of experiments are not possible. Interpretation of results obtained with the use of La must, therefore, be made with caution.

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